PATHOGENESIS OF THE GRAFT-VERSUS-HOST REACTION IN CHICK EMBRYOS

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STATEMENT

An examination of the vasculature of the normal chick embryo yolk sac (chapter 6) was carried out in association with Dr. G.I. Schoefl. Mr. R. Hill cut and stained the histological sections required for light microscopy. With these exceptions, the experimental work described in this thesis was carried out by myself.

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Candidate's signature.

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ABBREVIATIONS

B-lymphocyte	Bursa derived lymphocyte
CAM	Chorioallantoic membrane
EBM	Eagle's basal medium
GVH	Graft-versus-host
GVHR	Graft-versus-host reaction
MLR	Mixed lymphocyte reaction
NLT	Normal lymphocyte transfer
RNA	Ribonucleic acid
T-lymphocyte	Thymus derived lymphocyte

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CHAPTER 1

INTRODUCTION

CHAPTER 1. INTRODUCTION

The effect of adult lymphoid tissue on the developing embryo was first described by Murphy (1916) who showed that grafts of adult, allogeneic, lymphoid tissue placed on the chorioallantoic membrane (CAM) of a 7 day chick embryo caused spleen enlargement and the formation of semi-translucent tumour-like nodules on the CAM and on the surface of the spleen. Similar pathological conditions, involving proliferative changes in the lymphoid tissues of the host have now been described in many species (Simonsen, 1962; McBride, 1966).

It was found that these pathological changes only developed when adult lymphoid tissues were inoculated into a host which was unable to reject the donor cells (Simonsen, 1957). There are three types of situation where this may occur (Howard, 1963) : when the recipient is neonatal or embryonic and has not developed immune competence, when the recipient has acquired or inherited tolerance to the donor tissue, and when the immune capacity of the recipient has been depressed following treatments such as X-irradiation.

The development of the graft-versus-host concept

The idea of a graft-versus-host reaction (GVHR) arose from studies on the destruction of kidney homografts. Examination of this graft-host relationship led both Dempster (1951, 1953) and Simonsen (1953) to suggest that grafts containing lymphocytic elements might react against their hosts. Experimental evidence to support this suggestion was later independently provided by Simonsen (1957) and Billingham and Brent (1957).

When the GVH concept was proposed, the reaction was considered to involve a response by the donor cells to foreign transplantation antigens in the host (Simonsen, 1957). The donor cells were thought to proliferate within the lymphoreticular system of the host, attacking host cells by a homograft reaction and by the production of antibodies against host erythrocytes and possibly other tissues (Cock and Simonsen, 1958). The potential for a GVHR was therefore considered to depend on the presence of immunologically competent cells within the graft, and the presence of histo-

compatibility antigens within the host which were foreign to the donor tissue (McBride, 1966). If these conditions were fulfilled, the clinical expression of a GVHR then depended on the strength of the counterdirected host-versus-graft reaction (Howard et al., 1961; Howard, 1963, 1964).

The graft-versus-host reaction gained wide support as an explanation of the pathological condition which develops when adult lymphoid tissue is introduced into an allogeneic host which is unable to reject the donor tissue (Simonsen, 1962). Paradoxically, however, a reaction of the graft-versus-host type does not explain many of the pathological changes which occur in the host during the reaction (DeLanney et al., 1962). It also does not explain the inability for xenogeneic tissue to produce a reaction (Lafferty and Jones, 1969). Evidence which supports the interpretation that these pathological conditions are reactions of the graft against the host will now be considered.

Evidence in favour of the graft-versus-host concept

The nature of the effector cell

From as early as 1916, when Murphy described spleen enlargement in chick embryos grafted with adult tissue, it was known that not all tissues would produce this response in the embryo. Murphy found that while spleen, bone marrow, liver and kidney induced splenomegaly, pancreas, bone and muscle had no effect. Other adult tissues which were later shown to produce spleen enlargement when grafted into the chick embryo included thymus (DeLanney and Ebert, 1959a), blood (Simonsen, 1957), duodenum (Van Alten and Fennell, 1959) and skin (Billingham and Silvers, 1959a).

These tissues, however, are not all equally effective in eliciting splenomegaly. Cain and coworkers (1968) found that the injection of spleen cells into a 14 day chick embryo produces more spleen enlargement than the injection of the same number of thymus cells. Bursal cells gave virtually no spleen enlargement since any small increase in spleen weight they produced could be attributed to contamination of the bursa with cells from the blood.

All the tissues found to be capable of eliciting spleno-

megaly in the chick embryo were tissues which contained lymphoid elements (Simonsen, 1962). Other experiments demonstrated that the donor tissue had to contain viable lymphoid cells (Van Alten and Fennell, 1959; Mun et al., 1959) obtained from a donor in which immunological competence had developed (Seto, 1968). Tissue from embryonic or neonatal donors has little GVH activity (Ebert and DeLanney, 1960; Solomon, 1961) since immunological competence does not develop until late embryonic or early neonatal life (Seto, 1971; Solomon, 1971).

Simonsen (1957) and Cock and Simonsen (1958) had demonstrated that adult chicken blood could produce a GVHR in chick embryos. Terasaki (1959a) therefore attempted to determine the nature of the cell which was responsible for the initiation of a GVHR by fractionation studies of chicken blood. He showed that lymphocytes, but not monocytes, from fractionated blood, produced a GVHR in chick embryos.

At this stage, the most likely candidate for the immunologically competent cell, appeared to be a large dividing lymphocyte that would be capable of proliferation within the host (Gowans, 1968). Although evidence in favour of the large lymphocyte was put forward (Anderson et al., 1960; Cole and Garver, 1961; Szenberg and Warner, 1961, 1962), it was later conclusively shown from experiments with rat thoracic duct lymph (Gowans et al., 1961; Gowans, 1962) and from further fractionation studies with chicken blood (Solomon, 1964; Simons and Fowler, 1966; Szenberg and Shortman, 1966; Shortman and Szenberg, 1969) that the donor cell which initiated the GVHR was the small lymphocyte.

Initially, it was thought that the cell which was active in the GVHR was an immunologically competent cell from a functionally homogenous population (Medawar, 1963; Gowans et al., 1963). Experiments on neonatal thymectomy and hormonal bursectomy in the chicken (Warner et al., 1962) demonstrated, however, that there were 2 major lymphocyte populations; bursa-dependant lymphocytes and thymus-dependant lymphocytes. These two populations were found to have different functions in the immune response (Cooper et al., 1965, 1966; Warner, 1967). Thymus-dependant or

T-lymphocytes were involved in cellular immunity, 'immunological memory' and 'killer cell activity' (the ability of specifically sensitised cells to destroy cultured cells). Thymus-independant or B-lymphocytes, however, were responsible for antibody production (Roitt et al., 1969).

When T- and B-lymphocytes were investigated for their GVH activity, it was found that while spleen cells (Warner and Szenberg, 1963), or blood (Cooper et al., 1966), from an hormonally bursectomised chicken gave a normal GVHR in the chick embryo; blood from a neonatally thymectomised chicken showed a significant decrease in GVH activity (Cooper et al., 1966). Experimental evidence of this kind (Roitt et al., 1969) has demonstrated that the effector cell responsible for the initiation of the GVHR, is the thymus-derived or T-lymphocyte.

Genetic relation between host and donor

Donor cells which are syngeneic with the recipient are not able to produce a GVHR (Simonsen, 1957; Cock and Simonsen, 1958). It therefore appeared that a genetic difference between host and donor was required in order for a GVHR to take place. The best evidence, in support of this postulate (McBride, 1966) had been obtained from experiments in which parental cells were inoculated into their F_1 hybrid offspring. This inoculation produced a severe GVHR (Cock and Simonsen, 1958; Billingham and Brent, 1959). However, if F_1 cells were inoculated into neonatal parental recipients a GVHR did not result (Billingham and Brent, 1959). It was concluded that in the latter situation the direction of antigenic stimulation had been reversed allowing a host-versus-graft reaction to take place (McBride, 1966). Experiments of this kind provided strong support for the proposition that there must be a genetic and therefore antigenic difference between donor and recipient, in order for the graft to react against the host (Simonsen, 1962).

The genetic loci which control the GVHR have now been identified in several species (McBride, 1966). In the chicken, spleen enlargement (Jaffe and Payne, 1962) and pock formation (Burnet and Burnet, 1961) are controlled by the B locus (Schierman and Nordskog, 1963), the major locus res-

ponsible for tissue transplantation antigens in this species. Similarly in mice, the GVHR is controlled by the major H-2 locus (Billingham, 1966) and in rats by the Ag-B locus (Elkins and Palm, 1966).

Differences between host and donor at minor histocompatability loci are sufficient to initiate a homograft reaction, but they are not sufficient to cause a GVHR (Billingham, 1966). Minor differences between host and donor produce transient and trivial GVHR's (Simonsen, 1970a) and a strong GVHR only develops if host and donor differ at a major histocompatability locus. The GVHR therefore has more exacting genetic requirements than the equivalent homograft reaction (Billingham, 1966).

The identification of the effector cell which initiates a GVHR as a thymus-dependant small lymphocyte and the demonstration that a GVHR will only occur if there is a difference at a major histocompatability locus between the host and the donor, provides strong evidence that this condition involves some form of immune recognition between a thymus-derived small lymphocyte and the foreign tissue of the host. However, it has also been concluded from this evidence that the initiation of a GVHR depends on the stimulation of donor cells by foreign transplantation antigens of the host. Some anomalies which can not be explained by a reaction of this type will now be discussed.

Anomalous features of the graft-versus-host reaction

The nature of the host response

Although the pathology of the GVHR varies in different species, most descriptions include the development of widespread lymphoid tissue lesions (Simonsen, 1962). The host response is biphasic (McBride, 1966); an early proliferative phase, characterised by the destruction of lymphoid organisation and the proliferation of primitive reticular cells (Biggs and Payne, 1961, a, b) is followed by aplasia and atrophy of the lymphoid tissue (Billingham and Brent, 1959; Biggs and Payne, 1961b).

At first, the initial proliferative phase which led to spleen enlargement and pock formation in the chick embryo was considered to be the result of donor cell proliferation

within the lymphoreticular system of the host (Cock and Simonsen, 1958). The histological appearance of the enlarging spleen in chick embryos undergoing a GVHR, suggested, however, that both host and donor cells were involved in the proliferative response (Biggs and Payne, 1961, a, b; DeLanney *et al.*, 1962).

This conclusion was supported by studies in which karyological analysis (Biggs and Payne, 1959; Jaffe and Fechheimer, 1966; Weber, 1970) and radioisotopic labelling (Seto and Albright, 1965) were used to determine the contribution of host and donor cell proliferation to spleen enlargement. The proportion of donor and host cells which were found to proliferate in the spleen varied in different experimental conditions. Biggs and Payne (1959) found that 50% of the dividing cells in the enlarged spleen of 18 day old embryos, inoculated with adult blood at 14 days were of host origin and Jaffe and Fechheimer (1966) had similar results. Weber (1970), however, showed that the majority, if not all, of the dividing cells in the spleen and in pocks formed on the CAM from 2 to 6 days after the inoculation of allogeneic leukocytes into 12 day chick embryos, were of host origin.

Some of the variation in the proportion of donor and host cells proliferating in the spleen appears to depend on the age of the recipient. Nisbet and Simonsen (1967) found that if F_1 chick embryos were inoculated at 13 days with parental cells, most of the spleen enlargement which followed was due to the proliferation of host cells. In comparison, both host and donor cells were found to proliferate in the spleen of 17 day old recipients.

Similar karyological studies to determine the proportion of dividing host and donor cells in the spleen during a GVHR, have been undertaken in neonatal F_1 mice injected with parental lymphoid cells. Donor cell proliferation was found to occur in the initial stages of the reaction while most of the spleen enlargement in later stages was due to the proliferation of host cells (Davies and Doak, 1960; Howard *et al.*, 1961; Fox, 1962; Boraker and Hildeman, 1965). Although the experimental situation in neonatal F_1 mice is not directly comparable to that in the chick embryo, because of the effect

of embryo age on donor cell proliferation (Nisbet and Simonsen, 1967), studies in both species stress the importance of host cell proliferation in the development of splenomegaly. The proliferative lesions which occur in the host during a GVHR can thus no longer be attributed merely to the proliferation of stimulated donor cells within the tissues of the host. Many attempts have been made to explain this proliferative response on the part of the host, some of which will now be discussed.

Previous explanations of the host response

The early embryologists, who studied the effect of adult tissue on the developing embryo, considered that an adult graft altered the pattern of embryonic differentiation by stimulating certain elements in the host to proliferate. Murphy (1916) suggested that the adult graft stimulated the chick embryo spleen and caused proliferation of leukocytic elements in the mesoderm, skin, liver and kidneys. Danchakoff (1916, a, b; 1918, a, b), who undertook a histological investigation of the reaction produced by grafts of adult spleen in chick embryos, came to a similar conclusion. She thought that primitive mesenchymal elements in the host were directly stimulated by the graft towards granulopoiesis, an effect which was considered to support the theory that blood elements in the chick embryo had a monophyletic origin from primitive mesenchymal cells. Brandly and coworkers (1949) similarly stressed the increase in granulopoiesis, which occurred during 'embryo disease' produced in chick embryos by the inoculation of normal adult blood.

The effect of adult tissue on the development of the embryo was also examined by embryologists concerned with the problems of organ differentiation and growth. Weiss and Wang (1941) had shown that the liver of chick embryos became enlarged if pieces of adult liver were placed on the area vasculosa during development. Weiss (1950) subsequently suggested that organ growth was regulated by homologous cellular constituents which either acted as 'building blocks' or which had a 'template' or catalytic function. Ebert (1951, 1954) considered that this theory of organ specific growth stimulation might explain the phenomenon of spleen enlargement in chick embryos grafted with adult spleen. He

therefore carried out a series of radioisotope studies designed to show that subecellular units from the adult graft became incorporated within the homologous organ of the host and were responsible for its enlargement (Ebert, 1954).

After the concept of the graft-versus-host reaction had been formulated (Simonsen, 1957; Billingham and Brent, 1957) Ebert and his coworkers still maintained that the pathological changes which they described in the chick embryo could not be fully explained on the basis of immune mechanisms (Ebert, 1958, 1959; DeLanney and Ebert, 1959a; DeLanney et al., 1962). In place of the earlier building block hypothesis (Ebert, 1954) they introduced the concept of epigenetic recombination. Macromolecular components of the graft were considered to act, not as building blocks, but as centres of synthesis which redirected the course of differentiation towards granulopoiesis. They also suggested that the macromolecular components involved in this process might be RNA or RNA protein (DeLanney et al., 1962).

As the concept of the GVHR became firmly established these ideas were not favoured (Simonsen, 1957, 1962). However, the host cell proliferation was still not adequately explained (Nisbet and Simonsen, 1967). It did not appear to be immunological in nature (Fox, 1962) since it occurred in F_1 hosts, genetically tolerant of the donor parental strain cells. Various mechanisms for non-specific host cell proliferation during a GVHR have been proposed, including the suggestion that donor cells may release humoral factors which stimulate extramedullary haemopoiesis and enlargement of the host spleen (Elkins, 1971). Host cell proliferation has also been described as a mobilization reaction by the host in response to cellular injury caused by the donor cells (Seto, 1971). As yet, however, an adequate explanation for the host cell proliferation which occurs in the GVHR has not been obtained. It is a phenomenon which, as DeLanney and co-workers (1962) maintained, still cannot be fully explained in terms of a reaction in which the graft reacts against the host.

The effect of donor-preimmunisation on the graft-versus-host reaction

A reaction of the graft against the host was considered to result from the proliferation of donor cells following stimulation by foreign transplantation antigens of the host (Simonsen, 1957). If this is the case, preimmunisation of the donor might be expected to increase the severity of a GVHR by increasing the number of antigen-sensitive cells in the inoculum. Instead, it has been found that in the chicken, as in other species (Simonsen, 1970a; Ford and Simonsen, 1971), preimmunisation of the donor has no effect on the strength of the resulting GVHR, if host and donor differ at a major histocompatability locus (Lind and Szenberg, 1961). Preimmunisation of the donor will increase the severity of a GVHR if host and donor differ only at minor histocompatability loci (Warner and Szenberg, 1964).

Simonsen (1970a) has suggested that clones of antigen-sensitive cells for transplantation antigens governed by major histocompatability loci are fully expanded in the normal animal. In this situation preimmunisation of the donor would not increase the strength of a GVHR. Another explanation that must also be considered, however, is that antigen alone is not sufficient to initiate a reaction of the GVH type (Lafferty and Jones, 1969). Support for this proposition can be obtained from studies on the ability of xenogeneic tissue to produce a GVHR and on the tissue specificity of the reaction between host and donor cells.

The reaction of the host to xenogeneic tissue

Since the GVHR was first described (Murphy, 1916) it has been known that adult lymphoid cells have little or no GVH activity in a xenogeneic recipient (Brandly *et al.*, 1949; Ebert, 1951; Simonsen, 1957; Mun *et al.*, 1959; Isacson, 1959). Experiments on the response of chicken embryos to xenogeneic lymphoid tissue from different species has shown that a GVHR is only produced when there is a close phylogenetic relationship between donor and recipient (Mitchison, 1958; Payne and Jaffe, 1962; Lafferty and Jones, 1969). The GVHR, therefore, has more exacting requirements than the homograft reaction in this respect, since xenogeneic homografts are rapidly rejected (Billingham, 1966; Simonsen,

1970a).

The failure of lymphoid tissue to produce a GVHR in xenogeneic recipients has been attributed to the unsuitable nature of the host environment for the growth of the donor cells (Simonsen, 1962; Payne and Jaffe, 1962). Lafferty and Jones (1969), however, have shown that grafts of xenogeneic tissue can survive and proliferate in the chick embryo CAM, although they do not produce a GVHR unless the donor is preimmunised against chick embryo tissues. These experiments indicate that while a xenogeneic host provides a suitable environment for growth of donor cells, xenogeneic transplantation antigens do not present an appropriate stimulation for the initiation of a GVHR.

The failure of lymphoid cells to initiate a GVHR in xenogeneic recipients has been interpreted by Jerne (1971) as evidence which supports his theory of the generation of immune recognition. The theory postulates that during ontogeny, a large fraction of the lymphoid cells which arise in an animal are sensitive to the histocompatability antigens carried by other members of the species. Lymphoid tissue would therefore show a strong response to allogeneic tissue (allo-aggression) but a comparatively weak response to xenogeneic tissue. This hypothesis was considered to explain the relatively low number of cells required to produce a GVHR in the chick embryo (Simonsen, 1967; Nisbet et al., 1969) as well as the fact that xenogeneic tissue was ineffective in producing the reaction.

There is, however, an alternative explanation for this phenomenon. Since lymphoid cells can grow in an xenogeneic environment which is fully antigenic, without producing a GVHR, it has been suggested that antigen alone is not a sufficient stimulus for the initiation of a GVHR (Lafferty and Jones, 1969). This suggestion will now be examined in relation to the evidence on the tissue specificity of the interaction between host and donor cells in the initiation of a GVHR.

Tissue specificity of the interaction between host and donor cells

Evidence for the tissue specificity of the interaction between donor cells and cells of the host was first provided

by experiments on the normal lymphocyte transfer (NLT) reaction. This is an inflammatory response which was first described in the skin of guinea pigs after intradermal inoculation of allogeneic lymph node cells (Brent and Medawar, 1963). Since the NLT reaction showed the same specificity as a GVHR, it was considered to involve the reaction of donor lymphocytes against foreign histocompatability antigens of the host tissue (Brent and Medawar, 1966, 1967).

However, the inability of xenogeneic tissue to produce an NLT reaction (Jones and Lafferty, 1969; Jones et al., 1969) and the demonstration that the NLT reaction is histologically very different from dermal reactions produced by the interaction of antigen with sensitised cells (Jones et al., 1969) suggests that the NLT reaction, like the GVHR, can not be completely explained in terms of a response to antigen.

Further experiments which attempted to produce an NLT reaction in an irradiated host, have shown that the initiation of these reactions does not depend on an interaction of donor cells with foreign transplantation antigens, present on any host tissue, but must be initiated by a specific interaction between donor cells and the haematogenous cells of the host (Ramseier and Billingham, 1966). Ramseier and Billingham showed that heavily irradiated hosts developed only slight NLT reactions, even after the injection of very large numbers of donor lymphocytes.

Similar experiments have been carried out on the GVHR. As in the NLT reaction, irradiation of the host was found to prevent the development of local (Elkins, 1966) or generalised GVHR's (Seto and Albright, 1965). Elkins and Guttman (1968) also demonstrated the requirement for host cells of haematogenous origin in the development of the GVHR by injecting adult syngeneic spleen cells under the kidney capsule of a parental strain kidney which had been transplanted into an F_1 hybrid host. In this situation, where donor cells were confronted with an isogeneic kidney, perfused with allogeneic blood, a typical local GVHR (Elkins, 1964, 1966) developed under the kidney capsule.

The experiments of Streilein and Billingham (1970a, b) demonstrated that the requirement for host cells of haemato-

genous origin is genetically specific. They found that the GVHR in hamsters, which is characterised by a severe epidermolytic syndrome (Streilein and Billingham, 1970a) could not be produced in lethally irradiated F_1 hosts, reconstituted with bone marrow from parental donors treated with anti-lymphocyte serum, if the donor inoculum came from the same parental strain which had been used for the bone marrow reconstitution (Streilein and Billingham, 1970b). If, however, donor cells from the other parental strain were inoculated, a severe GVHR developed. It could therefore be concluded that host leukocytes which were allogeneic to the donor cells were required to produce the reaction.

Elkins (1971) has carried out a similar experiment in which parental cells were inoculated under the kidney capsule of X-irradiated, bone marrow reconstituted recipients. If the donor parental cells came from the same parental strain used for bone marrow reconstitution of the recipient, a GVHR did not develop. If, however, the donor cells were taken from the other parental strain, a typical local GVHR developed under the kidney capsule. These experiments again demonstrated that allogeneic haematogenous cells of the host are required for the initiation of a GVHR.

The experiments that have so far been described were carried out on neonatal or young recipients. Experiments on embryonic hosts, in which the leukocytes have not matured, indicate that in this situation the requirement for host cells is slightly different from older recipients. Lafferty and Jones (1969) found that chick embryos 'treated' with adult syngeneic leukocytes rapidly rejected grafts of bone, spleen or liver from allogeneic embryos, which were placed on the CAM, but were not able to reject grafts of embryonic heart muscle. If, however, the heart muscle was perfused with embryonic spleen cells before grafting, the heart muscle was rapidly destroyed. It therefore appeared that elements of the embryonic lymphoreticular tissue were required in the graft for the process of graft rejection to develop. The GVHR was similarly considered to require the presence of similar lymphoreticular elements within the host. An interaction between donor cells and host reticular tissue would then account for the characteristic distribution

of proliferative lesions within the lymphoreticular tissue (Simonsen, 1962).

The experiments which have been described demonstrate that reactions of the GVH type (Lafferty and Jones, 1969) involve an interaction between donor lymphocytes and host cells of haematogenous origin. If this is the case, the reaction can no longer properly be described by the term graft-versus-host, since a specific interaction between two cell populations is involved. A reaction which occurs in this manner, might therefore more accurately be described as a form of alloimmune responsiveness (Seto, 1971).

Although the requirement for host cells in the development of these reactions has been accepted, it is still argued that the source of stimulus for the donor cells is provided by the transplantation antigens on the surface of the haematogenous cells of the host (Ramseier and Billingham, 1966). This does not explain, however, why histocompatability antigens on the surface of haematogenous cells are effective while histocompatability antigens on the surface of other tissues are inadequate (Streilein and Billingham, 1970a, b).

In an attempt to explain this phenomenon, Elkins (1971) has suggested that host leukocytes are a better immunogen than other host tissues since they have a high mobility which would allow rapid contact with the donor cells and they are also a good source of histocompatability antigens (Manson et al., 1964). The requirement for host cells, however, is a continuing one (Elkins, 1966) since GVHR's do not propagate if transferred to a secondary irradiated host. This requirement remains difficult to explain unless the haematogenous cells of the host serve some function, other than, or in addition to, the simple presentation of antigen.

Further evidence on the role of antigen in the initiation of reactions of the GVH type, has been provided by experiments on the mixed lymphocyte reaction (MLR) which has been interpreted as the initial step in the development of an alloimmune response in vitro (Manson and Simmons, 1969).

The mixed lymphocyte reaction (MLR)

The mixed lymphocyte reaction, a proliferative response which occurs when 2 populations of allogeneic lymphoid cells are cultured together *in vitro*, was first described by Bain and coworkers (1964). It has since been investigated in several species (Dutton, 1967; Silvers *et al.*, 1967; Wilson, 1967; Wilson *et al.*, 1967; Kasakura and Lowenstein, 1967; Bach and Voynow, 1966) including the chicken (Alm, 1971). These studies have demonstrated that the specificity of the MLR is very similar to the specificity of the GVHR. Mixed populations of allogeneic lymphoid cells are required to produce the reaction (Wilson, 1967; Wilson *et al.*, 1967) and the cell which responds by proliferation in cell culture appears to be the thymus-derived small lymphocyte (Wilson *et al.*, 1967; Alm, 1971). The reaction is controlled at a major histocompatability locus (Wilson, 1967; Silvers *et al.*, 1967; Dutton, 1967) although in some cases a reaction can be produced with populations of lymphoid cells which differ only at multiple minor loci (Häyry and Defendi, 1970). As in the case of the GVHR, xenogeneic cells normally have little activity in a MLR (Lafferty and Jones, 1969; Wilson and Nowell, 1970; Wilson and Fox, 1971) although Daguillard and Richter (1970) have reported that human lymphocytes will stimulate rabbit lymphoid cells in culture.

Since the MLR has this characteristic specificity it is considered to provide a valid model for the events which occur in reactions of the GVH type (Lafferty and Jones, 1969). In an interpretation, that is analogous to the GVH concept (Simonsen, 1957) it has been assumed that lymphocyte proliferation in a MLR is the result of stimulation by foreign transplantation antigens present on other lymphocytes in the culture (Wilson, 1967; Wilson *et al.*, 1967; Main *et al.*, 1967). There is some evidence that antigen alone can stimulate cells in a one-way MLR (Viza *et al.*, 1968; Manson and Simmons, 1969). This evidence, however, can not be considered as conclusive since stimulation could not always be produced.

Other evidence has demonstrated that the presence of foreign transplantation antigens is not, in itself,

sufficient to produce a MLR. Hardy and Ling (1969) have shown that high concentrations of allogeneic erythrocytes or erythrocyte stromata, irradiated or disrupted Hela cells, disrupted lymphocytes, or microsome preparations from the spleen are all inactive in a MLR. Schellekens and Eisjvogel (1970) have similarly shown that human lymphocytes treated with metabolic inhibitors or with heat could not stimulate untreated lymphocytes in a MLR even though they retained at least 2 of their HL-A antigens on the cell surface.

Although transplantation antigens alone, or transplantation antigens on the surface of non-viable lymphoid cells do not appear to produce a MLR, it can still be argued, that inactivation of lymphocytes may qualitatively change the surface antigens so that they are no longer presented in a manner which is immunogeneic (Schellekens and Eijsvogel, 1970). This explanation, however, does not readily explain the inability of xenogeneic lymphoid cells to stimulate in a mixed lymphocyte reaction (Lafferty and Jones, 1969; Wilson and Nowell, 1970; Wilson and Fox, 1971).

Further evidence which suggests that antigen alone is not sufficient to produce a MLR is provided by recent work of Maclaurin (in press) on the nature of the cell which stimulates in this reaction. It was found that lymph node cells, taken from a donor rat which had been neonatally thymectomised 6 weeks previously, were not able to stimulate allogeneic rat thymocytes in mixed culture. The cell responsible for stimulation in a MLR therefore appears to be the T-lymphocyte. This evidence suggests that the stimulus in a MLR is not transplantation antigen alone, since, although transplantation antigens appear to be represented on all lymphocytes, only the T-lymphocyte has the ability to stimulate in mixed culture.

The problem of the role of transplantation antigen in the initiation of a MLR and other reactions of the GVH type, however, may only be solved when antigen alone can be conclusively shown to produce a response, or when another mechanism for the initiation of these reactions can be demonstrated. There is already some evidence which favours the presence of a mechanism other than antigen, since it has been shown that RNA isolated from lymphoid cells will pro-

duce reactions in allogeneic recipients which are similar to those of the GVH type.

The effect of RNA extracted from lymphocytes on allogeneic recipients

The effect of RNA isolated from allogeneic lymphocytes or spleen cells after intradermal injection in sheep has been described by Jones and Lafferty (1968). RNA which had been isolated from syngeneic or xenogeneic lymphoid cells did not produce a dermal response. In comparison, if RNA from an allogeneic donor was injected intradermally, a strong dermal reaction was evoked. The strength of this reaction could be correlated with the intensity of the NLT reaction produced by normal lymphocytes from the same donor and it was also reported that histologically, the dermal reaction produced by allogeneic lymphoid RNA was similar to an NLT reaction.

Sharon and Swartz (1970) also found that pock formation on the CAM of developing chick embryos inoculated with adult lymphocytes could be altered by the presence of allogeneic RNA. Adult lymphocytes, incubated with allogeneic RNA isolated from adult spleen cells, and then inoculated on the CAM of isogeneic chick embryos elicited pock formation. Xenogeneic RNA or RNA extracted from adult muscle, however, had no effect.

These experiments suggest that reactions of the GVH type may be mediated by the transfer of RNA or RNA containing material between donor lymphocytes and haematogenous cells of the host (Lafferty and Jones, 1969).

The direction of stimulus in a graft-versus-host reaction

In the GVH concept (Simonsen, 1957), donor cells were considered to be stimulated by the presence of foreign transplantation antigens of the host. The direction of stimulation in this model is therefore from the host to the donor tissue. In comparison to this theoretical prediction, recent experiments on pock formation in the chick embryo (Killby, Lafferty and Ryan, in press) have demonstrated that the direction of stimulation in this reaction is from the donor to host and not from host to donor. It was found that although allogeneic lymphocytes treated with mitomycin C or with X-irradiation to prevent cell division, were in-

capable of producing a GVHR when placed directly on the chick embryo CAM, addition of embryonic spleen cells to the inoculum produced large numbers of pocks. Mitomycin C treatment of the embryonic spleen cells prevented the reaction.

It was also found that although donor cell division was not required to initiate pock formation the donor cells had to be viable in order to produce a reaction. Pock formation no longer occurred when the donor cells were given high doses of irradiation before incubation with embryonic spleen cells. This requirement for viable cells may thus explain why irradiated donor cells placed directly on the CAM do not elicit pock formation (Longenecker et al., 1970), since they may die before they contact the target tissue.

As the division of donor cells is not required for the initiation of a GVHR in the chick embryo, this reaction can no longer be interpreted as a response of donor cells to stimulation by foreign transplantation antigen. An alternative explanation (Killby, Lafferty and Ryan, in press) suggests that allogeneic lymphocytes directly stimulate haematogenous cells in the host, which then proliferate. If the basic immunological event is considered to be the differential recognition of 'self' from 'not self' by lymphoreticular tissue, a reaction of this type may still be considered as an immune reaction because a recognition of 'self' from 'not self' by lymphoid cells is involved. In the conventional description of an immune reaction, however, lymphoreticular tissue is considered to react specifically to antigeneic material (Burnet, 1969). This definition, therefore, can not include forms of alloimmune responsiveness such as the GVHR, which do not appear to be mediated by antigen (Lafferty and Jones, 1969; Killby, Lafferty and Ryan, in press). If the broader definition of an immune reaction, however, is accepted, it can only be concluded that not all immune reactions are necessarily mediated by antigen alone.

The characteristics of the host cell

Although a great deal is known about the reactive donor cell responsible for the initiation of a GVHR, the nature and

role of reactive host cells in these interactions is far less clear. The available evidence suggests that this host cell is part of the circulating leukocyte population (Streilein and Billingham, 1970a, b; Elkins, 1971) or in the case of embryonic systems, is associated with more primitive elements of the blood forming system (Lafferty and Jones, 1969).

The developing chick embryo provides a suitable system for the further analysis of the part played by haematogenous cells in the pathogenesis of the GVHR. Haemopoietic cells first develop in the blood islands of the embryonic yolk sac, where they give rise to erythrocytes, thrombocytes and cells of the granulocytic series (Danchakoff, 1908, 1916c; Edmonds, 1966). At a later stage in development, haemopoietic stem cells, or their early, more differentiated derivatives, leave the yolk sac in the circulating blood (Moore and Owen, 1965; Moore and Metcalf, 1970) to seed out and initiate haemopoiesis in other embryonic tissues. They first invade the thymus at 8 days (Moore and Owen, 1967b; Owen and Ritter, 1969) and are present in the spleen at 12 days, and slightly earlier in the bone marrow (Moore and Owen, 1965). The bursa is not invaded until around 14 days (Moore and Owen, 1966).

In the early embryo, haematogenous cells in the circulating blood are derived from the blood islands of the yolk sac; erythrocytes increase steadily in numbers from the second day (Lemez̆, 1964), thrombocytes appear at 3 days (Sugiyama, 1926) and neutrophils are present from the 4th or 5th day (Danchakoff, 1908; Sabin, 1921; Sugiyama, 1926). Other blood elements appear much later in the circulating blood, after the initiation of haemopoiesis in other blood forming tissues of the embryo. Eosinophils, basophils and lymphocytes are seen in small numbers from the 14th day, while monocytes do not develop until the 18th day (Sugiyama, 1926; Sandreuter, 1951; Lemez̆, 1964).

Chick embryos at different ages thus contain haematogenous tissue at different stages of development. If adult lymphocytes are therefore introduced into allogeneic chick embryos of various ages, it may be possible to correlate the pathological changes which occur during the resulting

GVHR with the development of the lymphoreticular tissue at this stage. This approach has been used in the experimental work which will be described in the following chapters.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2. MATERIALS AND METHODS

EXPERIMENTAL ANIMALS AND ANIMAL EXPERIMENTS

Fertile chicken eggs

Randomly bred fertile eggs and inbred eggs of the AA and CC strain were obtained from the Division of Animal Genetics, C.S.I.R.O.. These eggs were incubated at 38°C in humidified incubators.

Chickens

The chickens used in this study were randomly bred white leghorn chickens and birds from the highly inbred AA and CC strains. Inbred birds were hatched from fertile eggs supplied by the Division of Animal Genetics, C.S.I.R.O.. All birds were housed in single bird battery units and fed "layer pellets" ad libitum.

Transplantation of tissues into the chick embryo

Intracoelomic grafting

Chick embryos of 4 days of age were grafted with fragments of spleen tissue by the method of Dossel (1954). At 4 days the embryo lies on its side and the embryonic coelom is still in broad communication through the umbilical ring with the extraembryonic coelom. An incision is made in the chorion and the graft is then gently pushed with a fine glass rod, through the umbilical ring from the extraembryonic coelom into the embryonic coelom. After the graft was positioned the egg was sealed with elastic tape and wax and left in a humidified incubator at 38°C.

Intravenous injection of chick embryos

Chicken embryos were injected intravenously by the method of Beveridge and Burnet (1946). In this method the egg was candled and the position of a large allantoic vessel was marked with a soft pencil. A square of approximately 0.5 cm by 0.5 cm was drilled in the shell around this area. The square of shell was removed and a drop of paraffin was placed on the exposed shell membrane to make it transparent. As the egg was candled, a 25½ gauge needle fitted to a 1 ml plastic syringe was introduced into the vessel and a 0.1 ml suspension of adult leukocytes in Eagle's basal medium was injected into the allantoic vessel. This method was also used for injection of colloidal carbon

into the embryo.

Inoculation of the chorioallantoic membrane (CAM)

Chick embryos were inoculated on the CAM by the method of Lafferty and Jones (1969). In this method fertile chicken eggs of 5 days incubation or more were candled and the position of the air space was marked with a soft pencil. A mark was also made on the side of the egg at a place where the shell could be drilled without damage to large blood vessels. A small hole was drilled through the egg shell at the position marked on the side of the egg. Another hole was drilled through the egg shell and through the underlying shell membrane above the air space. A drop of sterile gelatine saline (3% gelatine in 0.9% sodium chloride solution) was placed on the hole at the side of the egg and the shell membrane was broken with a sterile needle to allow the gelatine saline to flow in between the two membranes. This 'drops' or separates the CAM from the shell membrane to create an artificial air space on the side of the egg. Cell suspensions were pipetted onto the 'dropped' CAM through the hole in the shell. The shell was then sealed with elastic tape and the eggs were incubated at 38^{o}C in a humidified incubator.

Inoculation under the shell membrane of the air space

Fertile chicken eggs of 5 or 6 days' incubation were candled and the positions of the air space and the embryo were marked with a soft pencil. A square of approximately 0.5 cm by 0.5 cm was drilled in the shell above the air space, on the side nearest to the embryo. The square of shell was removed and a hole was carefully torn in the shell membrane to expose the underlying extraembryonic membranes. The inoculum was pipetted slowly onto this region so that it seeped underneath the shell membrane and onto the underlying yolk sac. The shell was then sealed with elastic tape and wax and the eggs were incubated at 38^{o}C in a humidified incubator.

Bleeding of chickens

Blood was withdrawn from the wing vein of an adult chicken into a syringe containing an equal volume of Alsever's solution. The diluted blood was then used for

inoculation of chick embryos.

Buffy coat separation of leukocytes from chicken blood

Two ml volumes of a 1:1 dilution of adult chicken blood in Alsever's solution were placed in 15 ml centrifuge tubes, made up to 8 ml with Alsever's solution, and centrifuged at 300 g for 7 minutes. The leukocyte-rich layer was removed from the top of the packed blood cells and transferred to glass capillaries (internal diameter 3 mm, length about 10 cm). These were centrifuged at 300 g for 7 minutes. The white cell layer, usually 1-2 mm thick, was carefully removed from the top of the packed cells. The leukocytes were washed with 10 ml Alsever's solution and finally suspended in Eagle's basal medium at the required concentration. Using this method, 3 ml of blood will yield approximately 10^8 leukocytes.

Preparation of spleen cell suspensions

Chicken donors were killed by dislocation of the neck. The spleen was dissected out aseptically and placed in Eagle's basal medium. Chick embryo donors were removed from the shell into a sterile dish and cut free from the extraembryonic membranes. The spleen was dissected out and placed in Eagle's basal medium. To prepare spleen cell suspensions, the spleen was finely minced in a small volume of Eagle's medium with 2 scalpel blades.

Removal of blood samples from the chick embryo

A large allantoic vessel in the CAM of 12 to 13 day chick embryos was exposed under the shell membrane as described in the method for intravenous injection of chick embryos. With the egg being candled, 0.2-0.4 ml of blood were slowly withdrawn from the allantoic vessel with a 25½ gauge needle fitted to a 1 ml plastic syringe which was mounted on a micromanipulator.

Measurement of blood clotting time

0.2 ml of blood withdrawn from the allantoic vessel of a chick embryo was placed immediately in the bottom of a 15 ml glass centrifuge tube. The clotting time was estimated as the time required for the first visual evidence of a blood clot to appear in the sample.

Measurement of bleeding times

The bleeding time of small vessels in the CAM was measured by a modification of the method described by Edmonds (1968). Chick embryos of 13 days of age were candled and the position of the air space was marked. The shell above the air space was removed and the shell membrane was wetted wth physiological saline and gently removed. Using a dissecting microscope, a small vessel in the underlying CAM was crushed with watchmakers' forceps and the time noted with bleeding stopped.

Injection of yolk sac vessels for topographic studies

Chick embryos of 15 days incubation were used. The pointed end of the egg was supported on a ring of plasticene within a petri dish and the egg shell overlying the air space was removed. When the exposed shell membrane was flooded with warm physiological saline it became transparent and the underlying extraembryonic membranes could be seen clearly. A hole was made through to the amnion with blunt forceps, avoiding larger blood vessels. The embryo was then pulled out through this hole and placed on a plasticene platform adjacent to the egg so that the umbilical stalk was exposed. The yolk sac and the extraembryonic membranes remained within the egg. Under the dissecting microscope the vessels of the stalk were dissected free and the vitelline artery was canulated with tapered polyethylene tubing (no. SP 10, internal diameter 0.28 mm, external diameter 0.16 mm), fitted to a 1 or 2 ml plastic syringe with a 27 gauge needle. The canula was held in place with watchmakers' forceps since too much injury was caused to the fragile embryonic tissue when the canula was tied in. A small amount of physiological saline was injected to test the placement of the canula. The vitelline vein was then cut to provide an out-flow and infusion of the coloured gelatine mass started. When the injection was judged complete (usually 2-3 ml) the stalk was ligated and cut and the egg was placed in ice-cold saline to harden the gelatine mass. After some time, the yolk sac was removed, washed with cold saline to remove adhering yolk and pinned out on dental wax for fixation in neutral buffered formalin.

In some of the preparations a double injection mass was

used. The vascular bed was first filled with carbon coloured gelatine. A small amount of yellow Microfil injection compound or carmine-coloured gelatine was then over-injected to fill part of the arterial network.

CHEMICALS AND PREPARATION OF SOLUTIONS

All chemicals used were analytical reagent grade unless otherwise stated.

Alsever's solution

sodium citrate $Na_3C_6H_5O_7.2H_2O$	40.0 g
sodium chloride	21.0 g
glucose	102.5 g
10% solution citric acid	40 ml
double distilled water	5 litres

The pH was adjusted to 6.1 with citric acid and the solution was dispensed in 100 ml amounts. These were autoclaved at 10 lbs. for 15 minutes and stored at 4°C. To each 100 ml of Alsever's solution the following antibiotics were added :

penicillin	80 µ/ml
streptomycin	50 µg/ml
neomycin	50 µg/ml

Carmalum

carminic acid	1 g
aluminium potassium sulphate	10 g
distilled water	100 ml

The solids were dissolved by gently heating the solution which was then filtered. One ml of formaldehyde was added as a preservative.

Colloidal carbon

A colloidal carbon suspension, C11/1431a (Günter Wagner, Pelican Werke, Hanover) was used for intravenous injections and for colouring the gelatine injection mass. The preparation contains 100 mg carbon/cc, 4.5% fish glue and 1.3% phenol.

Eagle's basal medium (EBM)

EBM was prepared by the method used in the Department of Microbiology, John Curtin School of Medical Research, Australian National University. Two stock solutions were

stored at 4°C after sterilization by seitz filtering and were mixed before use. This method is a slight modification of the method given by Eagle (1959).

Solution I

Preparations 1 and 2 were made and mixed together. The materials in preparation 3 were dissolved in boiling water and the solution was cooled and added to preparations 1 and 2. Preparation 4 was then added and the volume was made up to 2 litres. This solution was sterilized by seitz filtration and kept at 4°C until used.

Preparation 1.

sodium chloride	136.0 g	magnesium chloride	1.6 g
potassium chloride	8.0 g	glucose	20.0 g
sodium hydrogen phosphate	3.2 g	calcium chloride	2.0 g
water to	500 ml		

Preparation 2.

L-arginine	420 mg	L-phenylalanine	330 mg
L-histidine	210 mg	L-threonine	476 mg
L-isoleucine	524 mg	L-valine	468 mg
L-leucine	524 mg	L-tryptophan	82 mg
L-lysine	730 mg	water to	500 ml

Preparation 3.

L-tyrosine	360 mg
L-cystine	240 mg
concentrated hydrochloric acid	1 ml
water to	200 ml

Preparation 4.

biotin	20 mg	thiamine	20 mg
choline	20 mg	riboflavin	2 mg
folic acid	20 mg	phenol red	400 mg
pantothenic acid	20 mg	penicillin	500 mg
pyridoxal	20 mg	streptomycin sulphate	500 mg
nicotinamide	20 mg		
water to	500 ml		

Solution II

5.84 g of L-glutamine was dissolved in 400 ml of water. The solution was then sterilized by seitz filtration and

stored at 4°C.

Final preparation

For final use solutions I and II were added to 880 ml of sterile water. The pH was adjusted to 7.4 with 30 ml of 1.4% sterile sodium bicarbonate.

Formic acid decalcifier

sodium citrate	10.6 g
distilled water	80.0 ml
100% formic acid	25.0 ml
40% formaldehyde	5.0 ml

The solution was made up and stored at room temperature.

Gelatine injection mass

A gelatine injection mass was prepared by a modification of the method given by Guyer and Bean (1953).

gelatine	5 g
potassium iodide	5 g
distilled water	100 ml

The gelatine was slowly dissolved in 100 ml of warm distilled water. The potassium iodide was then added. This solution was mixed 1:1 with colloidal carbon to give a black injection mass which was liquid at room temperature and solidified during fixation with formaldehyde.

Glycerol jelly

glycerol	10 ml
10% gelatine dissolved in distilled water	20 ml

A small thymol crystal was added to prevent bacterial growth.

Microfil

Microfil MV - 122, a yellow silicone rubber injection compound (Canton Biomedical Products, Swarthmore, Pennsylvania) was used to inject vascular beds for topographic studies.

Neutral buffered formalin

$Na\ H_2PO_4.2H_2O$	9.0 g
$Na_2HPO_4.2H_2O$	16.3 g
formaldehyde	200 ml
water	1,800 ml

The solution was made up and stored at room temperature.

Sodium chloride solution

A sterile solution of 0.9% sodium chloride in distilled water was used as physiological saline.

LYSOSOMAL ENZYME ASSAYS

Nomenclature

The enzymes which have been assayed in this study are numbered and named according to the recommendations of the International Union of Biochemistry on the nomenclature and classification of enzymes as given by Barrett (1969).

Acid phosphatase

EC 3.1.3.2, orthophophoric monoester phosphohydrolase.

Acid ribonuclease

EC 2.7.7.16, ribonucleate pyrimidinenucleotide-2'-transferase cyclizing.

β-Glucuronidase

EC 3.2.1.31, β-D-glucuronide glucuronohydrolase.

N-Acetyl-β-D-glucosaminidase

EC 3.2.2.30, β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase.

Preparation of plasma samples for enzyme assay

Blood from chick embryos was spun in 5 ml centrifuge tubes at 300 g for 10 minutes. The plasma was removed from the top of the packed blood cells with a drawn out pasteur pipette and either assayed at once or stored at $4^{o}C$ for a short time.

Determination of lysosomal enzymes

Acid phosphatase

The method used for the assay of acid phosphatase was based on the method developed by Bessey and co-workers (1946). The substrate para-nitrophenyl phosphate is hydrolysed by acid phosphatase in 0.05M citrate buffer, pH 5.0, to para-nitrophenol and inorganic phosphate. When alkali is added the enzyme hydrolysis stops and the yellow para-nitrophenate derivative is formed.

Preparation of solutions

Citrate buffer 0.05M, pH 5.0

citric acid	0.410 g
sodium citrate	1.125 g

Dissolved in 100 ml of doubly distilled water.

Substrate

82.5 mg of para-nitrophenyl phosphate was dissolved in 5 ml of 0.05M citrate buffer, pH 5.0. The final molarity of this solution is 5.5×10^{-3}M. The solution is stable for 1 week at 4°C.

Method for plasma samples

plasma	0.1 ml
0.05M citrate buffer	0.8 ml
substrate	0.1 ml

The plasma sample was incubated with the substrate in citrate buffer for 1 hour at 37°C. Four ml of 0.1N NaOH was then added. The free para-nitrophenol was read on a spectrophotometer at 410 nm against a distilled water blank. Sample blanks were prepared by adding the substrate after the alkali. Only fresh plasma samples could be used for these assays.

β-Glucuronidase

The assay used for β-glucuronidase was based on the method of Talalay and co-workers (1946). The substrate is phenolphthalein β-glucuronide which is hydrolysed by β-glucuronidase in 0.1M acetate buffer, pH 4.6, to liberate free phenolphthalein. The hydrolysis is stopped and the colour developed by adding alkali to give a final pH of 10.5-10.7.

Preparation of solutions

Acetate buffer 0.1M, pH 4.6

0.1M sodium acetate	49 ml
0.1M glacial acetic acid	51 ml

Alkaline glycine buffer 0.1M, pH 11.2

glycine	4.10 g
sodium chloride	3.16 g

Dissolved in 100 ml distilled water to which 3.0 ml of concentrated sodium hydroxide had been added (3 g NaOH

in 3 ml distilled water). The solution was made up to 200 ml with distilled water, adjusted to pH 11.2 with a few drops of concentrated hydrochloric acid and made up to 250 ml.

Substrate

25 mg of phenolphthalein glucuronide was dissolved in 5.0 ml of 0.1M acetate buffer to give a 0.01M final solution. This solution stored at 4°C was very stable.

Method for plasma samples

plasma	0.1 ml
0.01M acetate buffer	0.8 ml
substrate	0.1 ml

The plasma sample in acetate buffer was incubated with the substrate for 18 hours at 37°C. 2.5 ml of glycine buffer was then added. The free phenolphthalein was read on a spectrophotometer at 540 nm against a distilled water blank. Sample blanks were prepared by adding the substrate after the alkali. β-Glucuronidase is a very stable enzyme and assays could be carried out on frozen plasma.

N-Acetyl-β-D-glucosaminidase

The method used for the assay of this enzyme was derived from Leaback and Walker (1961), Vaes and Jacques (1965) and Aronson and de Duve (1968). Acetyl glucosaminidase activity is measured by hydrolysis of para-nitrophenyl glucoside in 0.1M citrate buffer pH 5.0. The reaction is stopped and the colour developed by the addition of alkali.

Preparation of solutions

Citrate buffer 0.1M, pH 5.0

0.1M sodium citrate	65 ml
0.1M citric acid	35 ml

Glycine-sodium chloride-sodium carbonate buffer, pH 10.7

glycine	1.996 g
sodium chloride	0.784 g
sodium carbonate	1.760 g

Dissolved in 150 ml distilled water, adjusted to pH 10.7 with 0.1N sodium hydroxide and made up to 200 ml with distilled water.

Substrate

13.69 mg of para-nitrophenyl-N-acetate-β-D-glucosaminide (Sigma) was dissolved in 5.0 ml of 0.1M citrate buffer to give an 8mM solution. This is very nearly a saturated solution, which is stable if stored at 0°C.

Method for plasma samples

plasma	0.1 ml
0.1M citrate buffer	0.8 ml
substrate	0.1 ml

The plasma sample was incubated with the citrate buffer for 2 hours at 37°C. Three ml of glycine-carbonate buffer was then added and the free para-nitrophenol was read on a spectrophotometer at 410 nm against a distilled water blank. Sample blanks were prepared by adding the substrate after the alkali.

Method for spleen tissue

The spleen was weighed and mechanically disrupted with a pasteur pipette in 0.01M citrate buffer, pH 5.0, containing 0.2% triton. 0.1 ml of this suspension was used for the assay as in the method given for plasma samples.

Method for yolk sac

The yolk sac was removed and washed in physiological saline to remove the yolk. It was then cut up with scissors and mechanically disrupted with a pasteur pipette in 10 ml 0.01M citrate buffer, pH 5.0, containing 0.2% triton. Five ml of this suspension was air dried at 110°C to estimate the dry weight of the yolk sac. The remaining yolk sac suspension was diluted ten-fold with 0.01M citrate buffer and a 0.1 ml sample was used for the enzyme assay as in the method given for plasma samples.

Acid ribonuclease

The method used for the assay of acid ribonuclease was derived from de Duve et al. (1955) and Vaes and Jacques (1965). The assay relies on the liberation of acid soluble low molecular weight oligonucleotides from high molecular weight ribonucleic acid (RNA) by ribonuclease at pH 5.0. The residual high molecular weight RNA and contaminating tissue proteins are removed from solution by precipitation

with perchloric acid containing uranyl acetate. Activity measurements are made by reading the optical density of the RNA-protein free supernatant at 260 nm.

Preparation of solutions

Citrate buffer 0.1M, pH 5.0

Prepared as given in the method for N-acetyl glucosaminidase.

Yeast RNA solution

Yeast ribonucleic acid (Sigma type III) 30.0 mg dissolved in 1.0 ml 0.1M citrate buffer. The substrate was made up fresh each day and stored at 4°C.

20% Perchloric acid, 0.025% uranyl acetate solution

70% perchloric acid 28.6 ml

Made up to 100 ml with distilled water. 250 mg uranyl acetate was added.

Method for plasma samples

Sample

plasma	0.1 ml
0.1M citrate buffer	0.8 ml
substrate	0.1 ml

Reagent blank

0.1M citrate buffer	0.9 ml
substrate	0.1 ml

Sample blank

plasma	0.1 ml
0.1M citrate buffer	0.9 ml

The reaction mixtures were incubated for 1½ hours at 37°C. After incubation, 1.0 ml of chilled perchloric acid-uranyl acetate solution was added and the mixture was allowed to stand at 4°C for 1 hour. The precipitate of protein and nucleic acid was removed by centrifugation. 0.5 ml of supernatant was diluted with 4.5 ml of distilled water and the optical density of this solution was read at 260 nm.

Enzyme activity

Acid phosphatase

The acid phosphatase activity of plasma samples has been expressed in this study in terms of mg para-nitrophenol released per hour by 0.1 ml plasma. The calibration curve for the conversion of optical density at 410 nm to mg para-

nitrophenol was obtained from the optical densities given by dilutions of a stock solution of para-nitrophenol.

Preparation of stock solution of para-nitrophenol

174 mg of para-nitrophenol was dissolved in 10.0 ml of 0.02N NaOH and made up to 250 ml with 0.02N alkali.

N-Acetyl-β-D-glucosaminidase

The enzyme activity of plasma samples was expressed as mg para-nitrophenol released per hour by 0.1 ml plasma using the calibration curve obtained for para-nitrophenol.

The enzyme activity of spleen samples was similarly expressed as mg para-nitrophenol released per hour per mg (wet weight) of spleen, and that of the yolk sac samples as mg para-nitrophenol released per hour per mg yolk sac (dry weight).

β-Glucuronidase

The enzyme activity of plasma samples was expressed as mg phenylphthalein released per hour per 0.1 ml plasma. The calibration curve for the conversion of optical density at 540 nm to mg phenolphthalein was obtained from the optical densities given by a stock solution of phenolphthalein.

Preparation of a stock solution of phenolphthalein (1 mg/ml)

phenolphthalein	100 mg
ethyl alcohol	100 ml

Acid ribonuclease

The activity of acid ribonuclease in plasma samples has been expressed in this study as the optical density at 260 nm per 0.1 ml of plasma.

PREPARATION OF TISSUES FOR LIGHT MICROSCOPY

Fixation

Tissues were fixed for at least 24 hours in neutral buffered formalin. Chick embryo yolk sac or CAM was pinned out flat on dental wax before fixation.

Decalcification

Tissues containing bone were decalcified after fixation for 24 hours in formic acid decalcifier.

Embedding in glycol methacrylate

Tissue for light microscopy was embedded in glycol methacrylate by the method of Ruddell (1967a, b).

Preparation of stock solutions

Stock solution A.

Glycol methacrylate	80 ml
2-Butoxyethanol	16 ml
Benzoyl peroxide	0.27 g

Stock solution B.

Polyethylene glycol	15 ml
N,N'-diethylanaline	1 ml

Embedding medium.

Stock solution A	21 ml
Stock solution B	0.5 ml

The stock solutions were kept at 4°C, warmed to room temperature before use and measured out in disposable 20 ml and 1 ml plastic syringes. The embedding mixture was prepared directly before use.

Dehydration

Solid tissues were dehydrated for half an hour in 30% acetone, 50% acetone, 70% acetone and 90% acetone and for 1½ hours (3 changes) in dry acetone stored over anhydrous copper sulphate. Yolk sacs or chorioallantoic membranes pinned out on dental wax were dehydrated for 1 hour in 30% ethanol, 50% ethanol, 70% ethanol and 90% ethanol and for 3 hours (3 changes) in 100% ethanol before they were removed from the dental wax.

Infiltration and embedding

Solid tissues were taken through at least 2 changes of 6 hours or longer in stock solution A in open specimen jars, since air inhibits polymerisation of glycol methacrylate.

Chorioallantoic membranes or yolk sacs were cut into pieces of suitable size for embedding and infiltrated in at least 2 changes of 6 hours in stock solution A. Tissues were embedded by placing them into polyethylene snap-on caps (2½ cm diameter) or silicone rubber moulds. The cap or mould was then filled with glycol methacrylate embedding mixture and covered with a square of polyester plastic, avoiding trapped air bubbles. A piece of glass and a small

weight were placed on top to prevent buckling. When polymerisation was complete, after about 1 hour at room temperature, the plastic sheet was peeled off and the block was prised out of the mould with strong forceps.

Sectioning and staining

Glycol methacrylate blocks were sectioned on a microtome with a steel knife at 2μ and were usually stained with an azure II/methylene blue mixture (Richardson et al., 1960). Some were stained with haematoxylin and eosin according to the method described by Pearce (1961).

Preparation of whole mounts

Normal yolk sacs

Normal yolk sacs were washed free of yolk in physiological saline, pinned out on dental wax and fixed in neutral buffered formalin. They were stained for 5 minutes in 0.1% osmium tetroxide, dehydrated in ethanol and prepared as whole mounts in Canada balsam.

Chorioallantoic membranes (CAMs)

Pieces of formalin fixed CAM, pinned out on dental wax, were stained with carmalum for 15 minutes, cleared in glycerol and mounted in glycerol jelly.

Injected yolk sacs

Yolk sacs injected with coloured gelatine, pinned out on dental wax and formalin fixed, were cleared in glycerol. The glycerol was then drained off and the preparation was covered with warm glycerol jelly which was allowed to set at 4^{o}C. More warm glycerol jelly was then added and a glass slide (3" x $2\frac{3}{4}$") was placed over selected areas of the membrane. After the glycerol jelly had again set, the yolk sac around the slide was cut away. The preparation was now turned over, the dental wax was removed and residual glycerol was blotted off with filter paper. Warm glycerol jelly was dripped onto the membrane and a second slide was mounted. When the glycerol jelly had set, the edges of the two slides were sealed with araldite (Selley's Chemicals Ltd.).

Embedding in plastic

After dehydrating through alcohol and clearing in xylol, tissues were infiltrated at room temperature for several days in uncatalysed Biopot (Science Aids (Aust.) Pty. Ltd.). They were then embedded in Biopot containing catalyst. Polymerised blocks were polished on wet and dry emery paper and buffed.

PREPARATION OF TISSUES FOR ELECTRON MICROSCOPY

Tissue for electron microscopy was fixed in a formaldehyde-glutaraldehyde mixture, slightly modified from Karnovsky (1965, 1967).

Aldehyde fixative: 2% paraformaldehyde, 2% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4, containing 0.025% $CaCl_2$.

1.4 g of paraformaldehyde was dissolved in 35 ml of 0.2M sodium cacodylate buffer, pH 7.4, by gently warming in a water bath. The solution was cooled and 2 ml of 70% glutaraldehyde (Ladd) was added. The solution was made up to 70 ml with distilled water, 0.154 ml of 1M calcium chloride was added and the pH of the final solution was re-adjusted to 7.4.

Osmium fixative: 2% osmium tetroxide in 0.1M sodium cacodylate buffer, pH 7.4.

A stock solution of 4% osmium tetroxide in distilled water was mixed 1:1 with 0.2M sodium cacodylate buffer, pH 7.4.

Fixation

Small pieces of tissue were fixed for half an hour at room temperature in the glutaraldehyde-paraformaldehyde mixture, and for a further half hour at 4°C. They were washed for 30 minutes in 0.1M sodium cacodylate buffer, pH 7.4, and post-fixed in osmium tetroxide at 4°C for 2½ to 3 hours. The tissues were then given 3 changes of 5 minutes each, in 0.1M sodium cacodylate buffer, pH 7.4.

Uranyl acetate block staining and dehydration

After staining with 2% aqueous uranyl acetate for 1 hour at 4°C, the tissues were washed in 30% ethanol for half an hour and dehydrated in half hour changes of 50%,

70% and 90% acetone followed by 3 half hour changes of dry acetone stored over anhydrous copper sulphate.

Infiltration

Durcupan (Fluka) was used for embedding tissues for electron microscopy and was made up shortly before use. Equal quantities of Durcupan A and B, containing 0.2 ml of catalyst (Durcupan C) per 10 ml resin were mixed thoroughly while warm (about 37°C). The dehydrated tissues were infiltrated at room temperature for half an hour each in ascending Durcupan-acetone mixtures (1:4, 2:3, 3:2, 4:1) and given two changes of 2 hours each in undiluted Durcupan at 37°C.

Embedding

Tissues were embedded in gelatine capsules or as flat mounts as follows : a small ring of polyethylene was placed on a glass slide covered with a sheet of polyester. The tissue was placed within the ring which was filled with Durcupan and then covered with another square of polyester. A glass slide and a small weight were placed on top. The blocks were polymerised at 45°C overnight and at 76°C-80°C for 48 hours.

Sectioning

Sections for electron microscopy were cut on an LKB ultrotome using glass and diamond knives. Thick sections for light microscopy were dried onto glass slides within a plastic (mylar) ring, stained with an azure II/methylene blue mixture (Richardson et al., 1960) and mounted in D.P.X. (Gurr). Thin sections for electron microscopy were picked up on uncoated copper grids and stained with lead citrate (Reynolds, 1963). Thin sections were examined in a Siemens Elmiscope I electron microscope.

GENERAL METHODS

Photomontage

Photographic prints, matched for composite pictures were torn in an irregular line along the overlapping edges. The underside of the photographic paper was pulled back from the edge of the tear with fine forceps and the prints were glued onto cardboard with spray adhesive.

Three dimensional representation of serially sectioned tissue

Thick sections were cut from a block of yolk sac tissue embedded for electron microscopy. Every 5th section was placed on a glass slide, stained with an azure II/ methylene blue mixture (Richardson et al., 1960) and then photographed. The contours of several blood vessels from each photograph were traced onto sheets of cellulose acetate. These sheets were stacked between thick glass plates in the order in which the sections had been cut. The reconstructed vascular segments were then photographed.

CHAPTER 3

INFLUENCE OF RECIPIENT AGE ON POCK FORMATION AND SPLEEN ENLARGEMENT IN THE CHICK EMBRYO

CHAPTER 3. INFLUENCE OF RECIPIENT AGE ON POCK FORMATION AND SPLEEN ENLARGEMENT IN THE CHICK EMBRYO

Introduction

One of the most commonly described events during the development of the graft-versus-host (GVH) reaction in the chick embryo, is the occurrence of proliferative lesions within the haemopoietic tissue of the host (Murphy, 1916; Danchakoff, 1916a, b; Simonsen, 1957; Lafferty and Jones, 1969). In fact, methods for quantitating the severity of GVHR's depend on the evaluation of these proliferative changes, either by assessing the degree of spleen enlargement (Simonsen, 1962) or by counting the number of pocks on the chorioallantoic membrane (CAM) (Burnet and Boyer, 1961; Coppleson and Michie, 1966).

Solomon and Tucker (1963) examined the effect of recipient age on the GVHR using splenomegaly as an index of reactivity. The injection of adult spleen cells into 6 day old embryos gave little spleen enlargement; thereafter the degree of spleen enlargement increased with recipient age, reaching a maximum at 13 days. They suggested that the absence of spleen enlargement in embryos injected at 6 days with adult lymphoid cells was due to the poor arterial vascularization of the spleen at this stage and that injected cells were therefore unable to reach the target organ, in this case the spleen.

Although the developing spleen does not receive an arterial supply until about the 12th day of incubation, it is connected to the vascular circuit from its earliest stage of development by a branch of the omphalomesenteric vein (Romanoff, 1960). Evidently the absence of spleen enlargement in the 6 day embryo cannot be adequately explained by postulating that a mechanical barrier prevents injected cells from reaching the splenic tissues. An alternative explanation should be considered.

Since the spleen enlargement produced in 11 to 14 day recipients is largely due to a proliferation of host cells (Nisbet and Simonsen, 1967), younger embryos may lack a cell population which is capable of proliferating in response to the presence of adult allogeneic cells.

Table 3.1

Spleen wet weight (mg) of randomly bred embryos 6 days after the inoculation of 0.1 ml of diluted adult AA blood on the CAM.

	Average spleen weight ± standard error				
Age at inoculation	5 days	6 days	8 days	10 days	14 days
Experimental group	7.5 ± 0.6	10.2 ± 0.9	17.5 ± 1.7	36.5 ± 4.4	45.1 ± 6.6
Control group	7.3 ± 0.5	7.2 ± 0.4	10.5 ± 0.5	12.9 ± 0.7	15.4 ± 0.9
Average increase above control level	0.2	3.0	7.0	23.6	29.7
Average percentage increase	2.7%	41%	66%	182%	183%
t-Test probability	0.71	0.0028*	0.0020*	0.0001*	0.0182*

*Significant at the 5% level.

In order to test this hypothesis, adult lymphoid cells were inoculated onto the CAM of allogeneic embryos at different stages of development. Both pock formation and spleen enlargement were used to assay the intensity of the GVHR produced. If a mechanical barrier prevents donor cells from reaching the spleen, the effect of recipient age on spleen enlargement might be expected to differ from the effect of recipient age on pock formation since in the latter case, donor cells are placed directly on the target tissue.

Experimental results

The effect of recipient age on spleen enlargement

To determine the age at which significant spleen enlargement first occurred, 0.1 ml of a 1:1 dilution of adult AA blood in Alsever's solution, was inoculated onto the chorioallantoic membrane (CAM) of 5 to 14 day old randomly bred chick embryos. Ten to 14 embryos were included in each age group. Control groups received 0.1 ml of saline. Six days after inoculation the spleens of the recipients were weighed.

As shown in table 3.1 the degree of splenomegaly increased steadily with recipient age. This increase was significant at the 5% level in recipients of 6 days incubation and older, but was not significant in 5 day recipients. The average percentage increase in spleen weight also increased with age until in recipients older than 10 days it appeared to reach a plateau level.

The effect of recipient age on pock formation on the CAM

In order to show that the lack of splenomegaly in early embryos involved more than simply a lack of contact with the target tissue, donor cells were inoculated directly onto the CAM. In the early embryo, this membrane grows very rapidly and by the 7th to 8th day of incubation has extended throughout the blunt half of the egg to reach the equator of the yolk sac (Romanoff, 1960). Therefore, even at early stages, there is little question of inoculated donor cells making contact with this tissue.

Randomly bred embryos of 5 to 14 days of age were inoculated, on the CAM, with 0.1 ml of a 1:1 dilution of adult AA blood in Alsever's solution. Eight to 18 embryos

were used in each age group. Six days after inoculation, the number of pocks on the CAM was counted and the results are presented in fig. 3.1A.

In recipients inoculated at 5 days, the pock count is very low and many of these embryos (66%) had no pocks at all on the CAM. In older recipients, pock counts increased with recipient age. These results indicate that pock forming ability develops in most randomly bred recipients at about 6 days of age.

This experiment was repeated using inbred CC recipients. However, of fourteen 6 day old CC recipients inoculated with 0.1 ml of a 1:1 dilution of adult AA blood in Alsever's solution, none survived for more than 4 days. No deaths occurred in the control embryos inoculated with 0.1 ml saline.

In a further experiment, CC embryos of 6, 8, 10 and 14 days of age were inoculated on the CAM with 0.1 ml of a 1:4 dilution of adult AA blood in Alsever's solution. Seven to 10 embryos were inoculated in each age group. These embryos were at the 29-30, 32-33, 35 and 37th stage of development according to the Hamburger-Hamilton classification (Hamburger and Hamilton, 1951). Six days after inoculation, the CAM of each recipient was examined for pock formation. The results are shown in fig. 3.1B.

No pocks had developed in any of the CAM's from 6 day recipients and very few were found on the CAM of 8 day recipients. In embryos inoculated at 10 days, the number of pocks dramatically increased. These results indicate that pock forming ability in inbred CC embryos develops between the 8th and the 10th day of incubation.

<u>The relation between pock formation and spleen enlargement</u>

Recipient age influenced pock formation and spleen enlargement in a remarkably similar manner as shown in fig. 3.1C. In randomly bred embryos inoculated on the CAM at 5 or 6 days with adult blood, not all embryos developed pocks on the CAM within the next 6 days. When the recipients were divided into two groups : those which had developed pocks and those which had not, it was apparent that embryos which had developed pocks also showed a significant degree of spleen enlargement compared to control embryos ($p<0.05$).

Fig. 3.1A The effect of recipient age on the average pock count on the chorioallantoic membrane of randomly bred embryos, 6 days after the inoculation of 0.1 ml of a 1:1 dilution of adult AA blood in Alsever's solution. Vertical bars show the standard error of the mean.

Fig. 3.1B The effect of recipient age on the average pock count on the chorioallantoic membrane of inbred CC embryos, 6 days after the inoculation of 0.1 ml of a 1:4 dilution of adult AA blood in Alsever's solution. Vertical bars show the standard error of the mean.

Fig. 3.1C The effect of recipient age on the average pock count on the chorioallantoic membrane and on the average increase in spleen weight above control levels in randomly bred embryos, 6 days after the inoculation of a 1:1 dilution of adult AA blood in Alsever's solution.

Fig. 3.1D Average spleen weights of control embryos, embryos which developed pocks on the chorioallantoic membrane and embryos which did not develop pocks on the chorioallantoic membrane, 6 days after the inoculation of a 1:1 dilution of adult AA blood in Alsever's solution onto the CAM of 5 or 6 day randomly bred recipients. Vertical bars show the standard error of the mean.

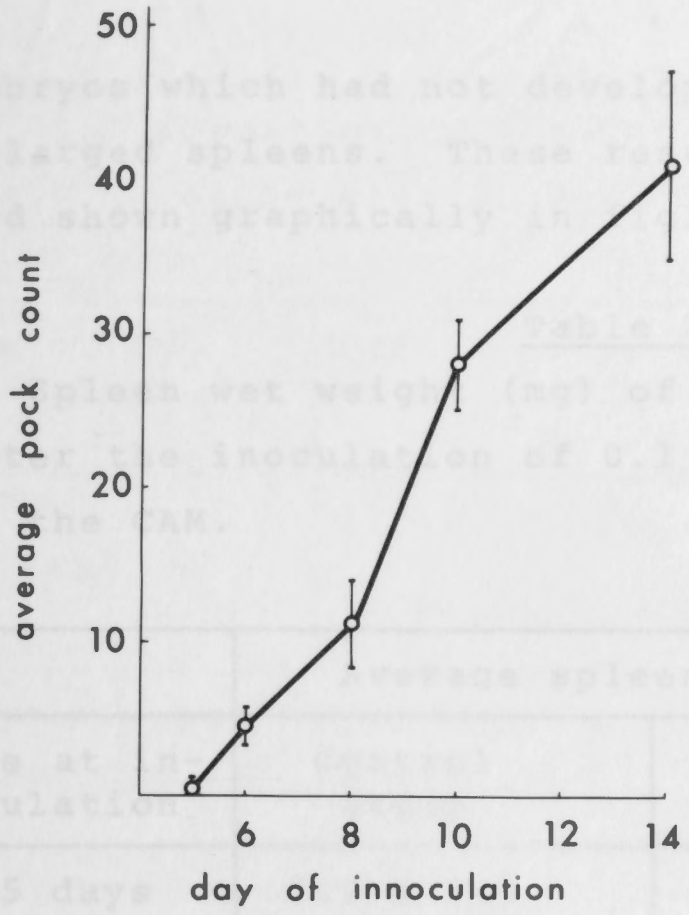


fig. 3.1 A

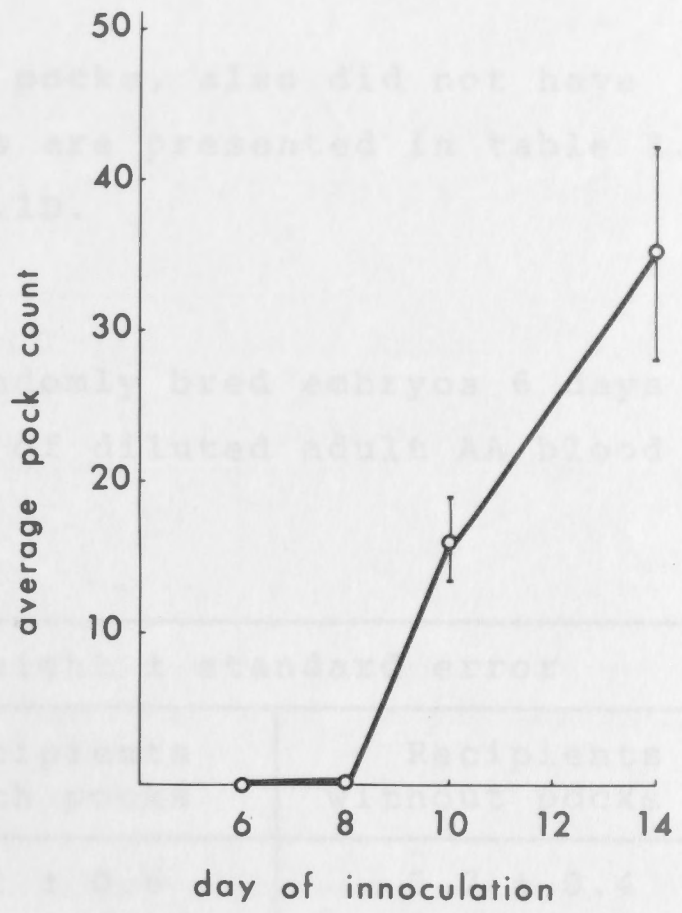


fig. 3.1 B

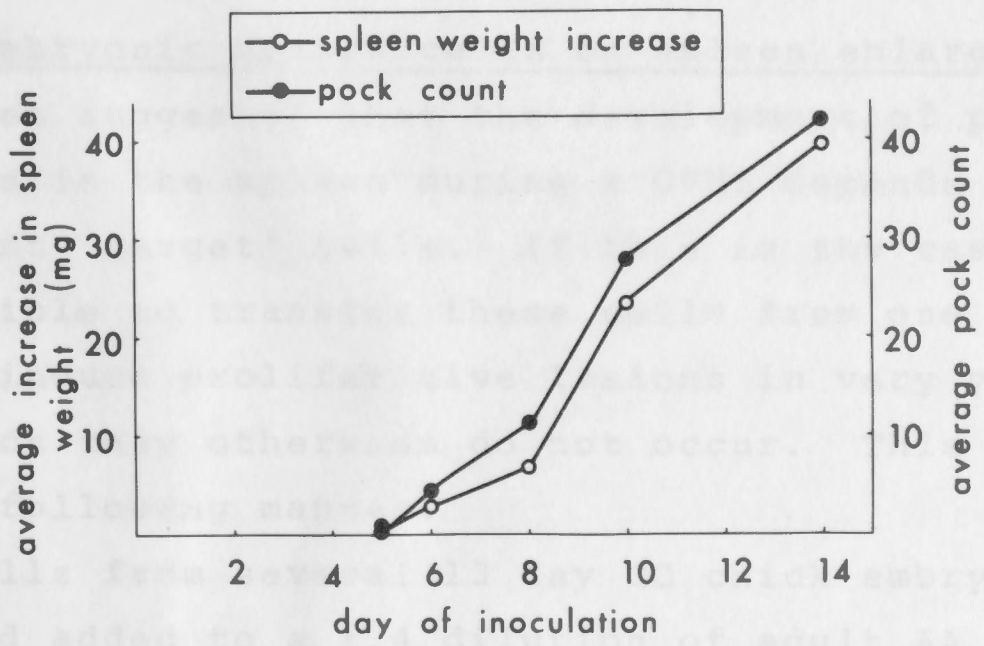


fig. 3.1 C

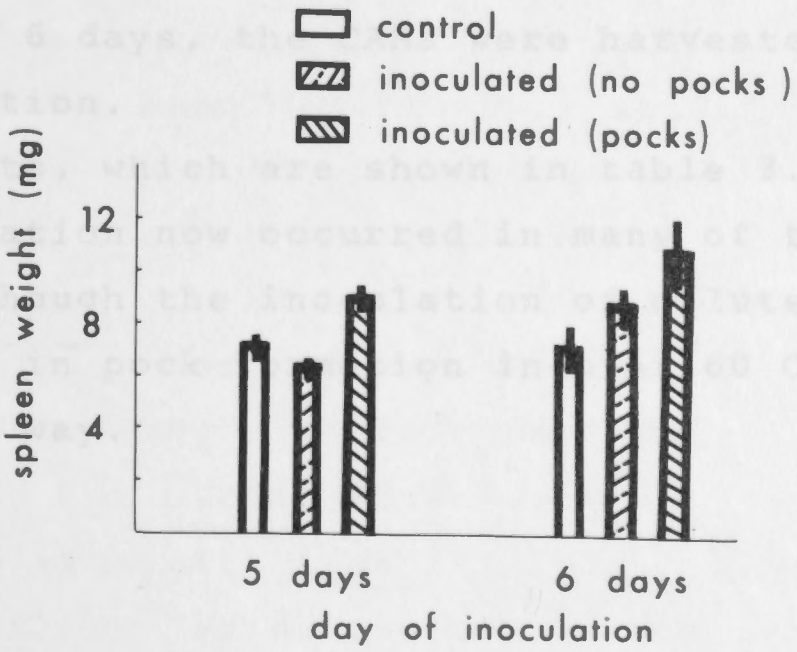


fig. 3.1 D

Embryos which had not developed pocks, also did not have enlarged spleens. These results are presented in table 3.2 and shown graphically in fig. 3.1D.

Table 3.2

Spleen wet weight (mg) of randomly bred embryos 6 days after the inoculation of 0.1 ml of diluted adult AA blood on the CAM.

	Average spleen weight ± standard error		
Age at inoculation	Control group	Recipients with pocks	Recipients without pocks
5 days	7.3 ± 0.5	9.2 ± 0.6	6.7 ± 0.4
6 days	7.2 ± 1.0	11.1 ± 1.4	8.8 ± 0.8

Influence of embryonic spleen cells on spleen enlargement

It has been suggested that the development of proliferative lesions in the spleen during a GVHR depends on the presence of host 'target' cells. If this is the case, it should be possible to transfer these cells from one embryo to another to induce proliferative lesions in very young embryos in which they otherwise do not occur. This was tested in the following manner.

Spleen cells from several 13 day CC chick embryos were pooled and added to a 1:4 dilution of adult AA blood in Alsever's solution. Each embryo received cells from 0.9 spleens from 13 day CC chick embryos in 0.1 ml of final inoculum. After 6 days, the CAMs were harvested and examined for pock formation.

The results, which are shown in table 3.3, indicate that pock formation now occurred in many of the 6 day CC recipients although the inoculation of diluted AA blood alone never resulted in pock formation in over 60 CC embryos tested in this way.

Table 3.3

Pock formation on the CAM of CC embryos inoculated with adult AA blood plus 13 day CC embryonic spleen cells*.

Age of inoculation	Number of pocks counted on each membrane	Mean count per membrane	% with pocks
6 days	0, 17, 0, 16, 7, 0, 0, 4, 4, 0, 2, 0, 2, 2, 4, 8, 2	4	64

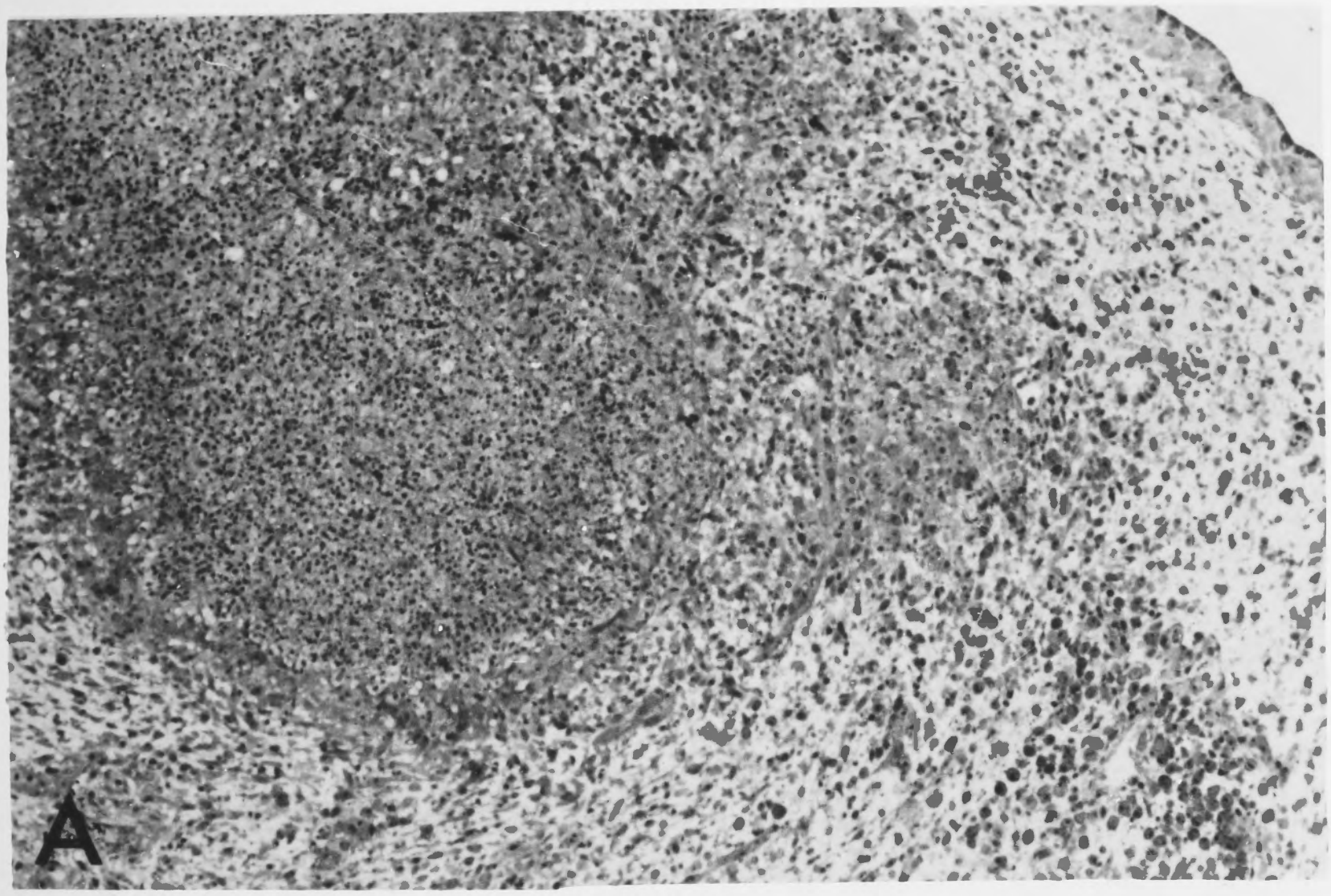
*Pooled results of 2 experiments carried out under the same experimental conditions.

The histological features of the pocks which formed on the CAM when embryonic spleen cells were added to the inoculum were examined in another experiment in which 8 day old CC embryos were inoculated on the CAM with 0.1 ml of a 1:4 dilution of adult AA blood in Alsever's solution to which spleen cells from several 15 day CC embryos had been added. Each embryo received 0.4 spleens from 15 day CC embryos. Eleven day embryos were similarly inoculated with 0.1 ml of a 1:4 dilution of adult AA blood only. Six days later, the membranes were harvested, fixed and embedded for light microscopy in glycol methacrylate.

In fig. 3.2, a section through a pock which formed in a 8 day CC embryo inoculated with allogeneic blood plus embryonic spleen cells (fig. 3.2A), is compared with a pock formed in the CAM of an older embryo, inoculated with allogeneic blood alone (fig. 3.2B). In each case, the pock consists of a focal aggregation of primitive cells, similar to those which have been described in splenic nodules during the development of splenomegaly (Biggs and Payne, 1961a, b). Pock formation in the CAM is, in both cases, accompanied by a general increase in the cellularity of the mesenchyme and a thickening of the chorionic epithelium.

Pock formation may therefore be induced in very early embryos which do not usually develop this lesion by the addition of spleen cells from an older embryo (in which pock forming ability has developed) to the inoculum of adult allogeneic blood.

Fig. 3.2 Proliferative lesions produced in the chorio-allantoic membrane when (A) adult AA blood plus 15 day embryonic spleen cells are introduced onto the CAM of an 8 day CC embryo and (B) adult allogeneic leukocytes alone are inoculated onto the CAM of 11 day old embryos.
Azure II/methylene blue Magnification 180 x.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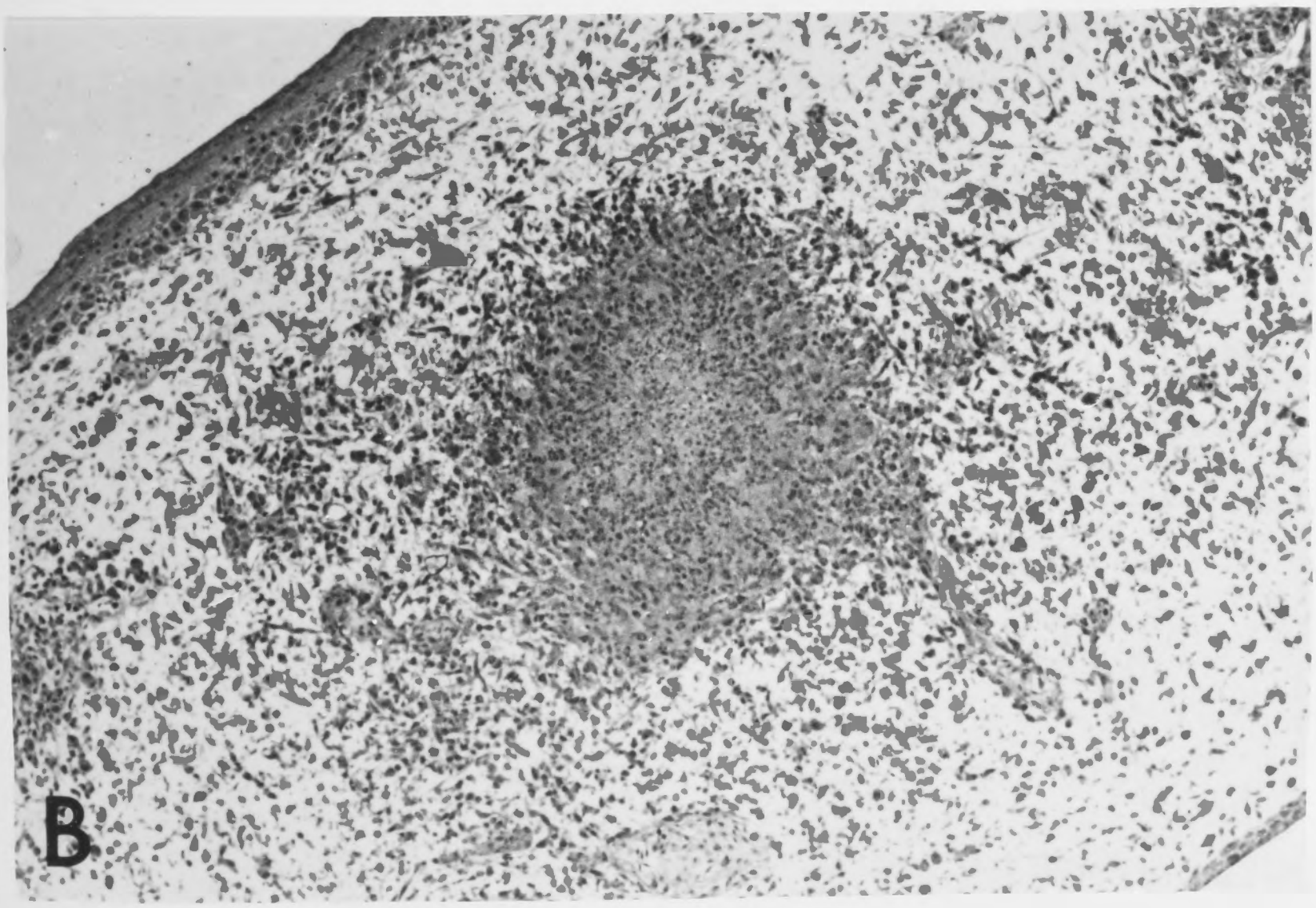

B

Discussion

While the GVHR was considered to result from the stimulation of donor cells by the foreign transplantation antigens of the host (Simonsen, 1957), the absence of spleen enlargement in very early embryos could be explained by the lack of antigenic stimulation provided by these hosts. This explanation, however, is inadequate, since studies on tolerance induction (Terasaki, 1959b; Hašek, 1960) have shown that transplantation antigens are present in the 4 or 4½ day chick embryo.

Since the lack of spleen enlargement in early embryos could not, therefore, be due to the absence of transplantation antigens in the early embryo, Solomon and Tucker (1963) suggested that donor cells might be unable to reach the spleen because of its poor arterial vascularization at this stage. The experiments which have been described above are not consistent with this hypothesis.

A re-examination of the effect of recipient age on spleen weight in randomly bred embryos showed that a definite age effect was operating. The results, however, are not completely in accord with those of Solomon and Tucker (1963) since they show that a significant increase in spleen weight ($p<0.01$) occurred before 15 days, i.e: in the 6 day recipients examined at 12 days. However, they do agree with the results of Ebert (1959) who found that when 7 day embryos were inoculated on the CAM, the first sharp increase in spleen weight occurred at 12 days.

A further examination of the effect of recipient age on pock formation showed that this effect was similar to that produced on spleen enlargement even though the donor cells were placed directly on the target tissue. If a mechanical barrier prevented donor cells from reaching the spleen, young embryos would be expected to show pock formation but little spleen enlargement.

The fact that there was a correlation between the effect of recipient age on pock formation and on spleen enlargement supports the hypothesis that the absence of proliferative lesions in very young embryos relates to an absence of host 'target' cells. This is further supported by the experiments in which pocks could be induced in very

young recipients if the inoculum of adult blood contained spleen cells from embryos which normally would develop proliferative GVH reactions.

It is now well established that host cell proliferation makes an important contribution both to spleen enlargement (Nisbet and Simonsen, 1967) and to pock formation (Weber, 1970) in the chick embryo. The host cells involved appear to be elements of the reticular tissue (Lafferty and Jones, 1969). It has also been shown that the activation of these haematogenous cells of the host during a GVHR is due to a specific interaction between allogeneic host and donor cells and is not merely a non-specific response (Streilein and Billingham, 1970b).

Haemopoiesis in the chick embryo originates with the development of haemopoietic stem cells of the area vasculosa and then in the yolk sac. Certain haemopoietic stem cells from the yolk sac later migrate into the haemopoietic tissues of the embryo at certain stages of embryonic life (Moore and Owen, 1965, 1966, 1967a, b). These stem cells are first detected in the tissues at around 8 days incubation (Moore and Owen, 1967a; Owen and Ritter, 1969) when they enter the thymic anlagen, other haemopoietic tissues are populated subsequently (Moore and Owen, 1965, 1967b). It is notable that pock forming ability develops about the time that haemopoietic stem cells first appear in the embryonic circulation. Proliferative changes in the chick embryo during a GVHR may therefore depend on the presence of stem cells, or their more differentiated derivatives within the lymphoid tissues of the host and in the embryonic circulation. Since it has been demonstrated (Killby, Lafferty and Ryan, in press) that the mere presentation of antigen on the surface of host 'target' cells is not sufficient to initiate pock formation, a direct interaction between allogeneic donor cells and suitable 'target' cells of the host may be involved. The experiments described in subsequent chapters were designed to show whether other aspects of the pathogenesis of the GVHR in the chick embryo were consistent with such an hypothesis.

CHAPTER 4

THE DEVELOPMENT OF HAEMORRHAGIC LESIONS DURING THE GRAFT-VERSUS-HOST REACTION IN VERY YOUNG CHICK EMBRYOS

CHAPTER 4. THE DEVELOPMENT OF HAEMORRHAGIC LEGIONS DURING THE GRAFT-VERSUS-HOST REACTION IN VERY YOUNG CHICK EMBRYOS

Introduction

The experiments described in chapter 3 have shown that proliferative changes such as spleen enlargement and pock formation on the CAM, which characterise the GVHR in older chick embryos (Simonsen, 1962) do not occur in very young recipients. This may be due to the absence of a GVHR in these early embryos (Solomon and Tucker, 1963). However, it is also possible that the expression of the GVHR is of a different nature. Support for this suggestion comes from experiments on the chick embryo (Ebert, 1957) and salamander (DeLanney, 1958) which demonstrated that, at an early stage of embryonic development, the GVHR may have a profound effect on the vascular bed.

When adult spleen was transplanted into the coelom of 4 day chick embryos (Ebert, 1957), at a time when the host spleen is no more than a mesenchymatous ridge, an extensive breakdown of the vascular bed occurred leading to haemorrhage and large areas of stasis in the extremities. Similarly, the grafting of adult spleen into the body wall of young salamanders (DeLanney, 1958), at a stage before the spleen is formed, resulted in a high mortality, transient polycythemia and areas of vascular stasis over most of the body surface.

The following experiments were carried out to determine whether the absence of proliferative lesions in the early chick embryo was due to the absence of a GVHR.

Experimental results

Intracoelomic grafting of adult spleen tissue

In a series of experiments similar to those described by Ebert (1957), adult spleen tissue was introduced into the coelom of 4 day old chick embryos.

Preliminary experiments indicated that a very high mortality occurred with this method of grafting. When adult CC spleen fragments were grafted into 20 AA recipients at 4 days incubation, only 2 survived for 5 days. These 2 survivors showed extensive haemorrhages.

Three experiments were then carried out in which spleen fragments from a randomly bred adult donor were grafted into the coelom of 4 day old CC recipients. These embryos were examined at 3, 4 or 6 days after grafting for the presence of haemorrhages. The results of these experiments are shown in table 4.1. Embryos in which most of the body surface was covered with petechial haemorrhages are termed 'severely' haemorrhaged, others in which only small regions of the body were involved, are termed 'slightly' haemorrhaged.

Table 4.1

The mortality and extent of haemorrhages in 4 day old CC chick embryos with intracoelomic grafts of adult spleen tissue from a randomly bred donor.

	Days after grafting		
	3 days	4 days	6 days
Number of embryos grafted	18	16	18
Number of embryos surviving	9	3	2
% mortality	50%	83%	89%
Number with haemorrhage	9	3	2
Severity of haemorrhage	slight to severe	slight to severe	severe

Histologically, no cellular proliferation with the formation of reticular cell foci (Biggs and Payne, 1961a, b) was seen in the liver and spleen of the surviving recipients in these 3 experiments.

To determine whether haemorrhages could only be induced with allogeneic tissue, small fragments of spleen from a 12 day CC chick embryo donor were grafted into the coelom of 4 day old syngeneic embryos which were examined 6 days later. None showed any sign of haemorrhage (5/5) indicating that these lesions can only be induced by allo-

geneic spleen cells. However, only a small number (20%) of the recipients survived for 6 days, many had died within 24 hours after grafting. Since this suggested that the high mortality in previous experiments was not solely due to the development of haemorrhagic lesions, but could also be attributed to operational trauma and to the degree of inbreeding in these recipients, other methods of grafting were tried.

Intravenous injection

Forty three CC embryos at 6 days of age were injected intravenously with 5×10^5 adult AA lymphocytes. Thirty seven per cent were alive 6 days later, 3 embryos had severe, the others slight haemorrhages. No evidence of cellular proliferation was seen in sections of the spleen.

The degree of spleen enlargement and the incidence of haemorrhage were compared in syngeneic and allogeneic recipients injected intravenously, at 6 days, with 1×10^7 adult AA leukocytes. Six days later all surviving CC recipients (9/9) had developed haemorrhages but all the surviving AA recipients (10/10) were entirely normal. Thus the development of haemorrhages depends on a genetic difference between host and donor and appears to have the same specificity as a GVHR in older embryos that is characterised by proliferative lesions.

The spleen weights of allogeneic and syngeneic recipients in this experiment were compared with the average spleen weight of normal 12 day CC embryos. The results are shown in fig. 4.1. The average spleen weight of allogeneic recipients was significantly lower than that of syngeneic recipients ($p<0.01$). Both were lower than the average spleen weight of normal uninoculated CC embryos. Lack of spleen enlargement in young chick embryos inoculated with adult allogeneic leukocytes correlates with the absence of pock forming ability in embryos of this age (see chapter 3).

Since haemorrhagic lesions have only been reported in embryos of up to 6 days, older embryos were tested in another experiment. Ten day old CC chick embryos were injected intravenously with 10^7 adult AA leukocytes. Six days after injection, the average spleen weight of these

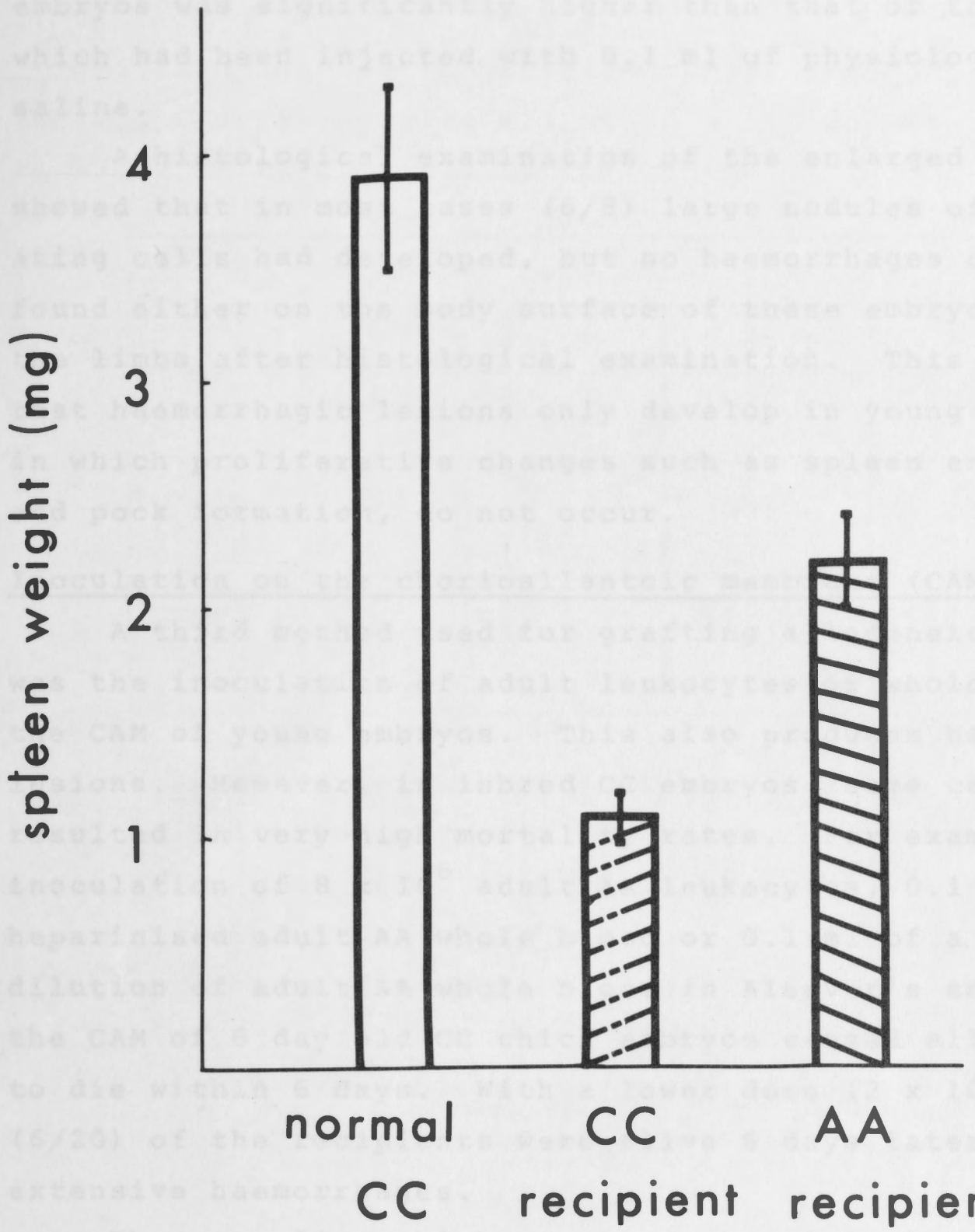


Fig. 4.1

The average spleen weight of normal 12 day CC chick embryos, 12 day AA embryos injected at 6 days with 1 x 10^7 adult leucocytes cells and 12 day CC embryos injected at 6 days with 5 x 10^5 adult AA buffy coat cells. Vertical lines indicate the standard error of the mean.

embryos was significantly higher than that of the controls which had been injected with 0.1 ml of physiological saline.

A histological examination of the enlarged spleens showed that in most cases (6/8) large nodules of proliferating cells had developed, but no haemorrhages could be found either on the body surface of these embryos, or in the limbs after histological examination. This suggests that haemorrhagic lesions only develop in young embryos in which proliferative changes such as spleen enlargement and pock formation, do not occur.

Inoculation on the chorioallantoic membrane (CAM)

A third method used for grafting allogeneic tissues was the inoculation of adult leukocytes or whole blood onto the CAM of young embryos. This also produces haemorrhagic lesions. However, in inbred CC embryos, some cell doses resulted in very high mortality rates. For example, the inoculation of 8×10^6 adult AA leukocytes, 0.1 ml of heparinised adult AA whole blood or 0.1 ml of a 1:1 dilution of adult AA whole blood in Alsever's solution on the CAM of 6 day old CC chick embryos caused all recipients to die within 6 days. With a lower dose (2×10^5), 30% (6/20) of the recipients were alive 6 days later. All had extensive haemorrhages.

The mortality which resulted when 0.1 ml of a 1:1 dilution of adult AA blood in Alsever's solution was inoculated on the CAM of 6 day old CC recipients was very variable. In one experiment, 83% (16/19) of the recipients survived for 6 days, while in another only 18% (3/17) survived for the same length of time. In most experiments, however, mortality and incidence of haemorrhage increased sharply with time.

This can be seen from the results of 3 experiments in which 6 day old CC embryos were inoculated on the CAM with 0.1 ml of diluted adult AA blood. The embryos were examined either 1, 2 or 3 days after inoculation. The results of these experiments are presented in table 4.2. There were over 20 embryos in each experimental group.

Table 4.2

Mortality and extent of haemorrhage in 6 day old CC chick embryos inoculated with 0.1 ml of a 1:1 dilution of adult AA blood in Alsever's solution on the CAM.

	Days after grafting		
	1 day	2 days	3 days
% mortality	17%	28%	38%
% with haemorrhage	33%	80%	100%
Severity of haemorrhage	slight	slight to severe	slight to severe

These experiments show that haemorrhages can be induced in chick embryos of 4 to 6 days incubation when they are grafted with adult allogeneic lymphoid tissue. The method by which the donor tissue is introduced has little effect on the development of these lesions. Haemorrhages did not appear when embryonic tissue or adult lymphoid tissue from a syngeneic donor was grafted. The pathological changes observed therefore appear to be the result of a GVHR.

Haemorrhagic lesions

Macroscopic appearance

In the normal embryo, the period from 4 to 12 days is one in which many tissues and organs differentiate. At 4 days, the brain, the eyes, and the branchial arteries are well developed, the allantois has appeared and the intestinal tract is beginning to differentiate. By 10 days, most organ systems are approaching their definitive form and the embryo has a distinctly avian appearance (Arey, 1957).

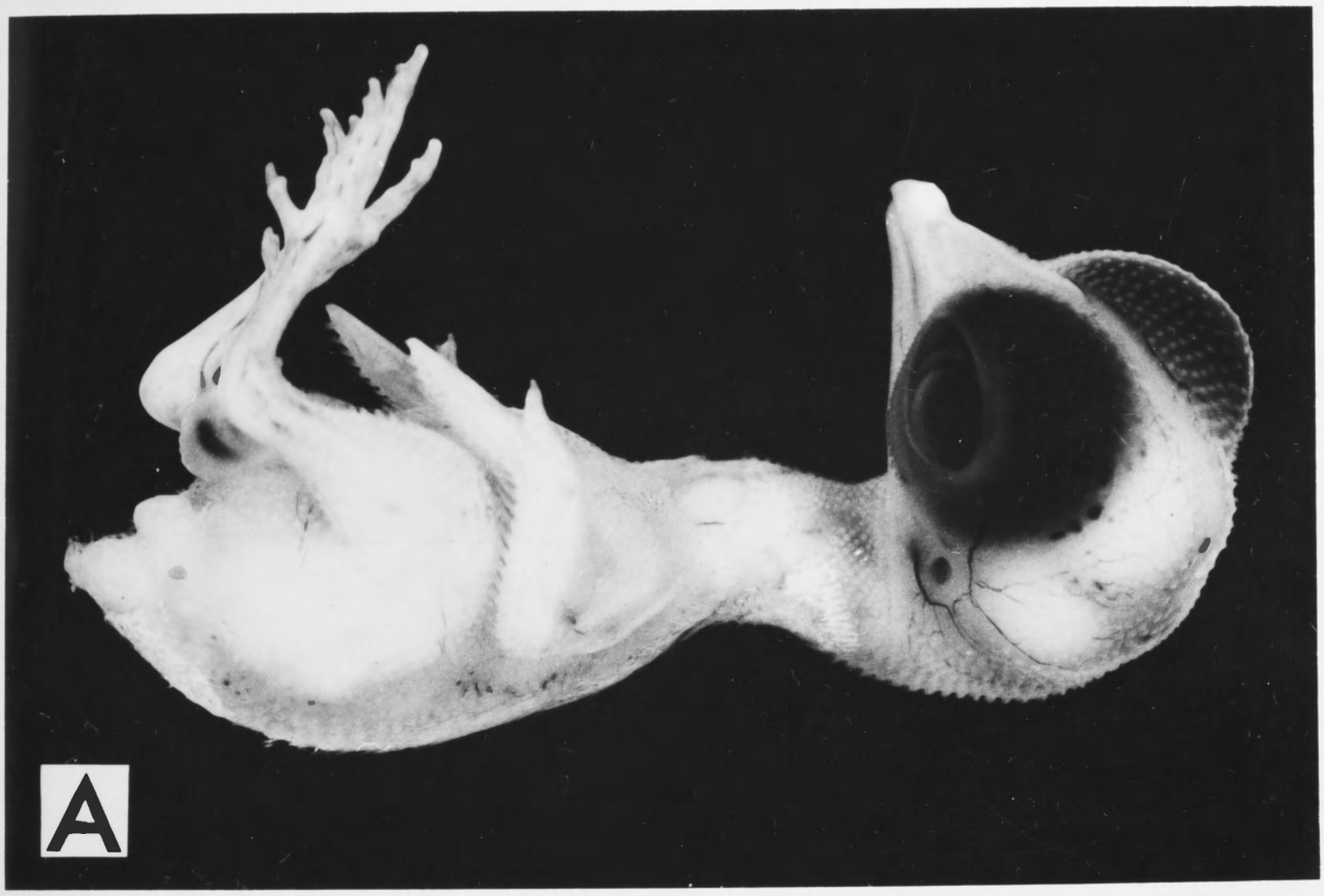
A normal 12 day CC embryo is shown in fig. 4.2A. At this stage, the embryo has well developed limbs and a distinct neck. The feather germs are conspicuous, especially on the wings, and the gap between the lower and upper eyelid is relatively small. These features are characteristic of an embryo at the 38th stage of incubation on the

Fig. 4.2A A 12 day normal CC chick embryo.

Magnification 2½ x.

Fig. 4.2B A 12 day CC chick embryo inoculated at 6 days on the CAM with 0.1 ml of a 1:1 dilution of adult AA blood in Alsever's solution.

Magnification 6 x.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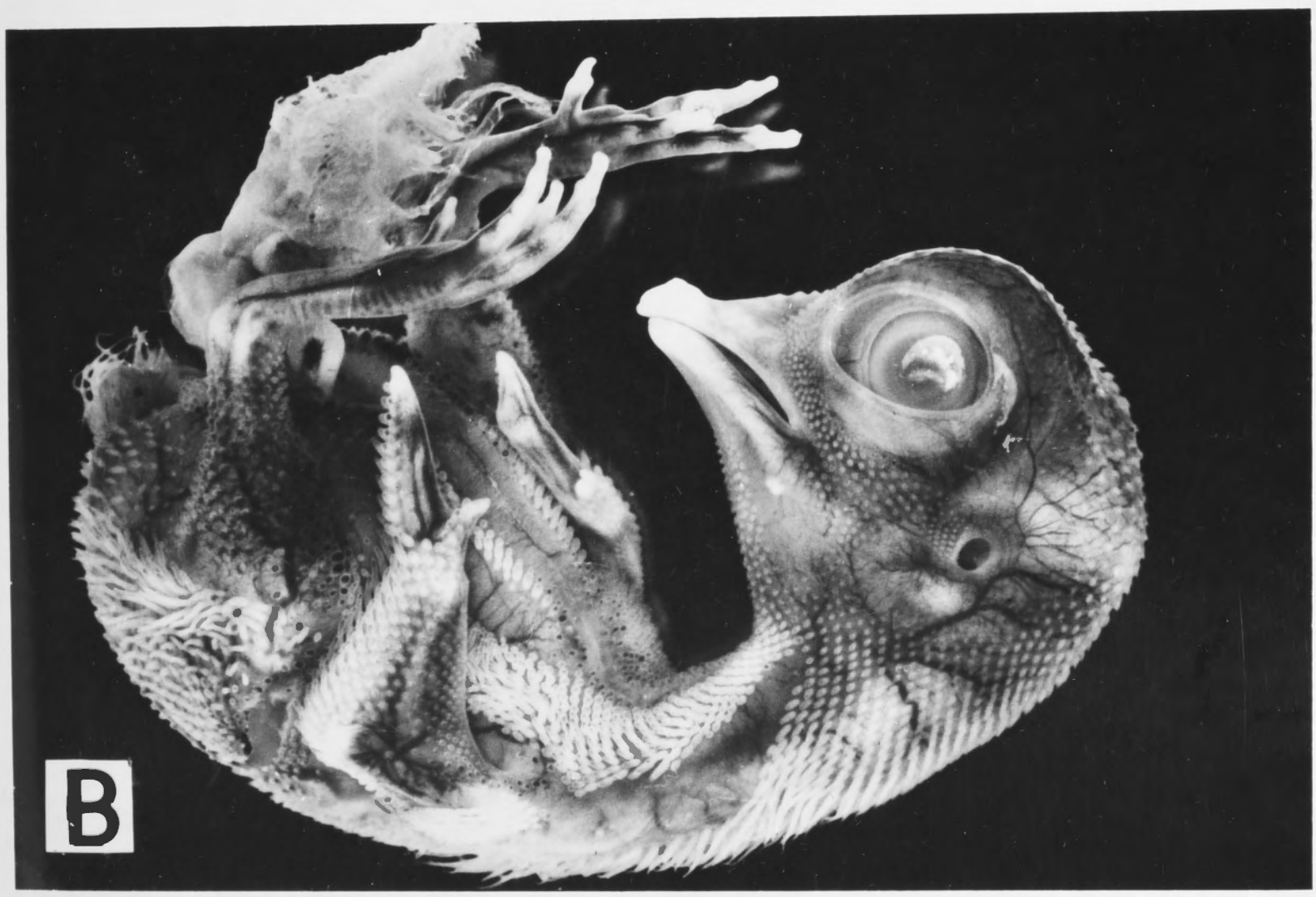

B

Hamburger-Hamilton scale (Hamburger and Hamilton, 1951).

Fig. 4.2B shows a 12 day CC embryo after inoculation of 0.1 ml diluted adult AA blood on the CAM at 6 days of incubation. This embryo is shown at approximately 3 times the magnification of the normal embryo. It can be seen that it is much smaller and less well developed. The limbs, feather germs and eyelids are characteristic for an embryo at the 37th stage of incubation on the Hamburger-Hamilton scale (Hamburger and Hamilton, 1951). The blood vessels in the skin are abnormally prominent and appear to be congested with red blood cells. Multiple petechial haemorrhages are present all over the body surface, in some areas discrete, as on the digits of the hind legs, in others confluent, as on the wings.

In these embryos haemorrhages occurred on the CAM as well as on the body surface. Fig. 4.3A shows a whole mount of the CAM from a 12 day CC embryo which has been inoculated on the CAM at 6 days with adult allogeneic blood. Petechial haemorrhages occur in many of the vessels.

A histological examination of the haemorrhagic lesions, as they occur in the middle digit of the hind limb, and in the CAM, was made by light and electron microscopy.

Blood vessels in the hind limb of normal embryos - light microscopy

The blood vessels of the limb buds first develop as a dense capillary network that grows into the limb bud at 3 days incubation (Evans, 1909). At 4 days, the entire blood supply comes from this capillary network. By 10 days, the definitive circulation has formed (Romanoff, 1960) and is supplied by the sciatic artery (Zackerkandl, 1895). The period during which haemorrhages can be induced by allogeneic lymphoid tissue therefore encompasses the period during which the definitive circulation becomes established in the hind limb.

The extent of the vascular bed in the middle digit is shown in fig. 4.3B. The embryo was injected with colloidal carbon through an allantoic vein (chapter 2). Since the tissue has been cleared, only the blood vessels filled with carbon are visible. The digital artery supplies an extensive capillary network throughout the digit which is drained

Fig. 4.3A Whole mount of the CAM of a 12 day CC chick embryo inoculated on the CAM at 6 days with 0.1 ml of a 1:1 dilution of adult AA whole blood in Alsever's solution. The arrows indicate small petechial haemorrhages in the vessels of the CAM.

Carmalum Magnification 6 x.

Fig. 4.3B Digit from the hind limb of a 10 day normal CC chick embryo, injected with colloidal carbon and embedded in plastic.

Magnification 4 x.


A


B

by the digital vein. A transverse section through such a digit is shown in fig. 4.4A. The central cartilage in which calcification is progressing (Fell, 1925) is surrounded by a wide layer of connective tissue which contains developing muscle bundles and an extensive network of thin walled, undifferentiated capillary vessels. The digit is covered by an epithelial layer, several cells thick.

Figs. 4.4B-E compare at a higher magnification, sections from the middle digit of the hind limb from normal CC embryos 8 to 14 days old. There is little difference, at the light microscope level, in vessels from the 8 day digit to vessels in the 14 day digit. They are thin walled capillaries surrounded by stellate mesenchymal cells of the connective tissue layer.

Blood vessels in the hind limb of normal embryos - electron microscopy

Sections from the middle digit of the hind limb of 6, 10 and 12 day CC embryos were examined by electron microscopy. A vessel from the hind limb of a 6 day embryo is shown in fig. 4.5A. At this stage, the vessels are lined by a low, continuous endothelium with no underlying basement membrane. In some areas, the endothelial cells are very attenuated and processes from neighbouring cells may overlap for some distance. The distribution of mesenchymal cells around the vessel is irregular and periendothelial cells are not present. Mesenchymal cells may contact each other by short, blunt cytoplasmic processes. At this stage, there are very few collagen fibres in the intercellular space.

Mesenchymal cells have a large nucleus and the cytoplasm contains many polyribosomes interspersed among short profiles of rough endoplasmic reticulum. Round or tubular mitochondria and large spherical opaque inclusions which resemble fat droplets may also be seen. The endothelial cell cytoplasm is similar in appearance to that of the mesenchymal cells. The nucleus is oval and irregular in outline with clumped areas of chromatin.

A vessel from the middle digit of the hind limb of a normal 10 day chick embryo is shown in fig. 4.5B. The endothelium is surrounded by well developed periendothelial

Fig. 4.4A Photomontage of a transverse section through the middle digit of the hind limb of a normal 10 day embryo embedded in glycol methacrylate and stained with azure II/ methylene blue. The section shows the normal structure of blood vessels (bv), connective tissue (ct), muscle bundles (m) and the central cartilage (cr) in the digit at this stage.

Magnification 110 x.

Fig. 4.4B-E Transverse sections to show the structure of vessels in the digit of 8 day to 14 day chick embryos. The tissue was embedded in Durcupan and sections were stained with azure II/methylene blue.

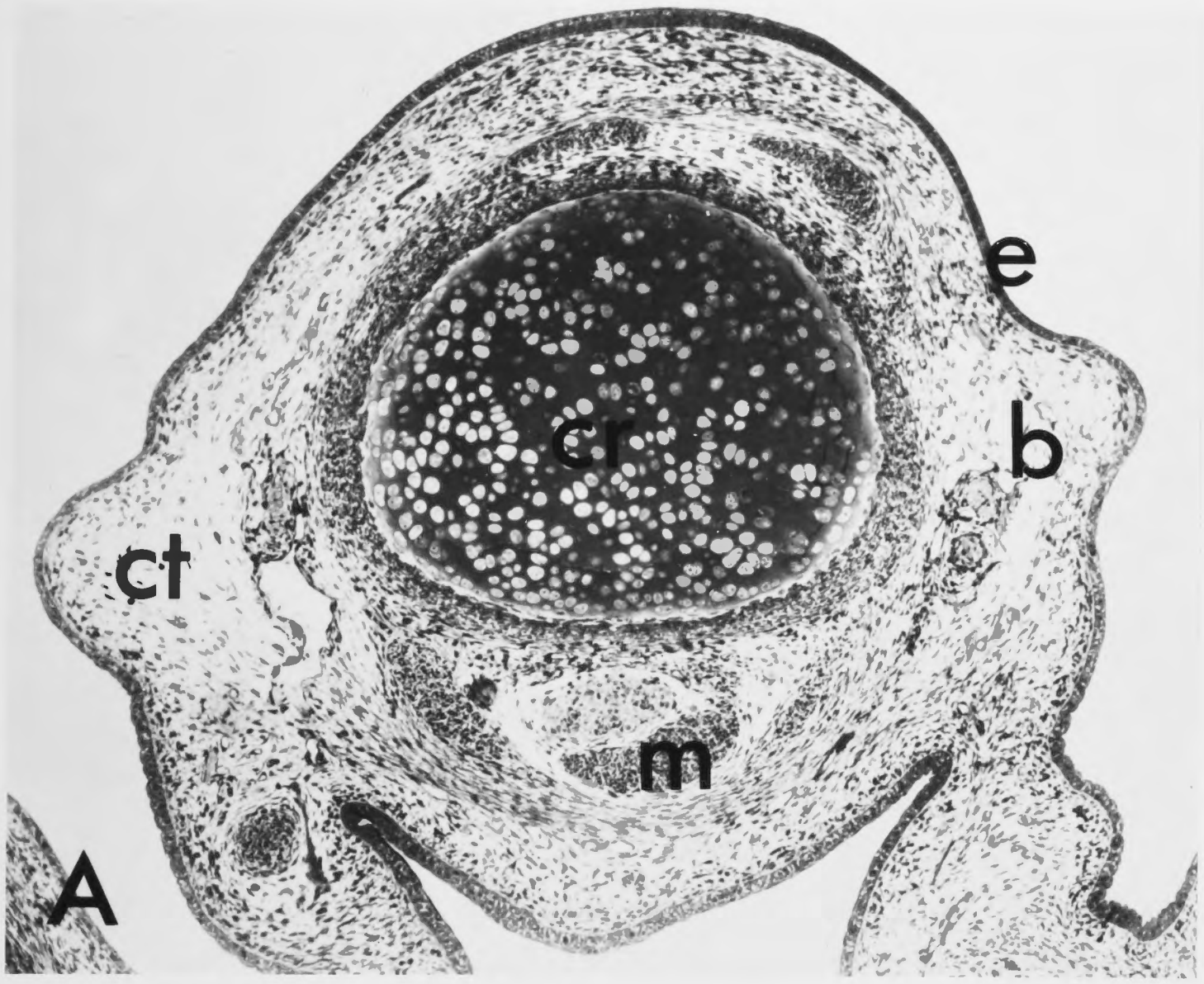
Magnification 200 x.

Fig. 4.4B 8 days

Fig. 4.4C 10 days

Fig. 4.4D 12 days

Fig. 4.4E 14 days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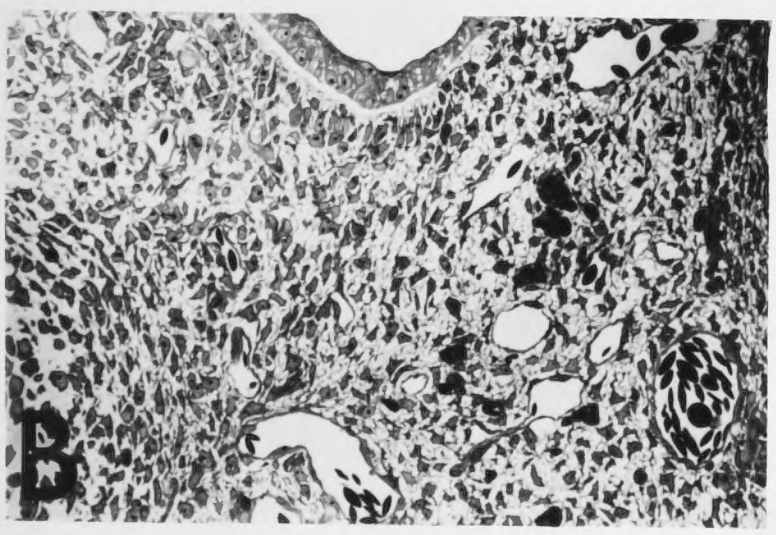
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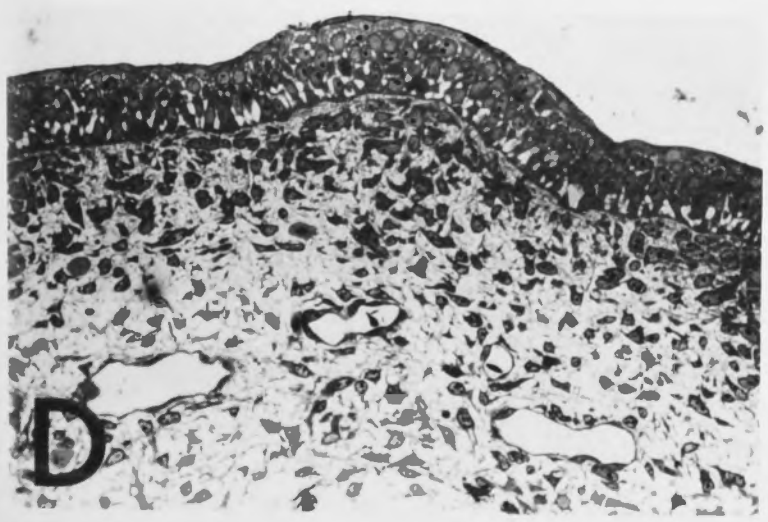
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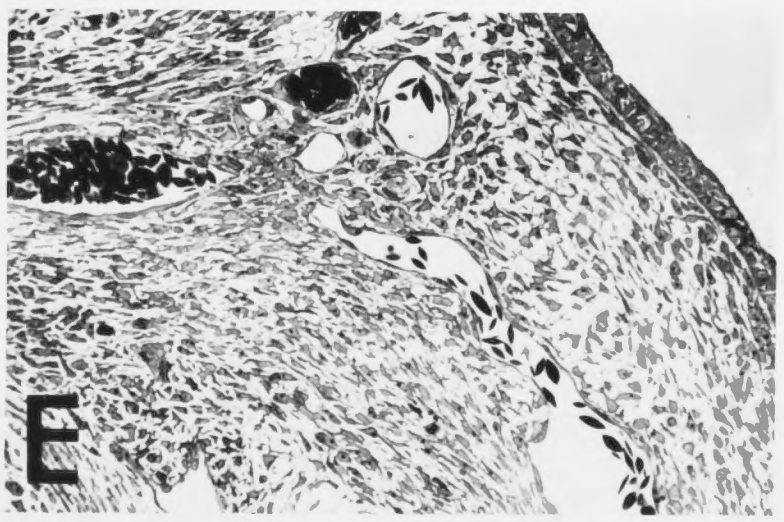
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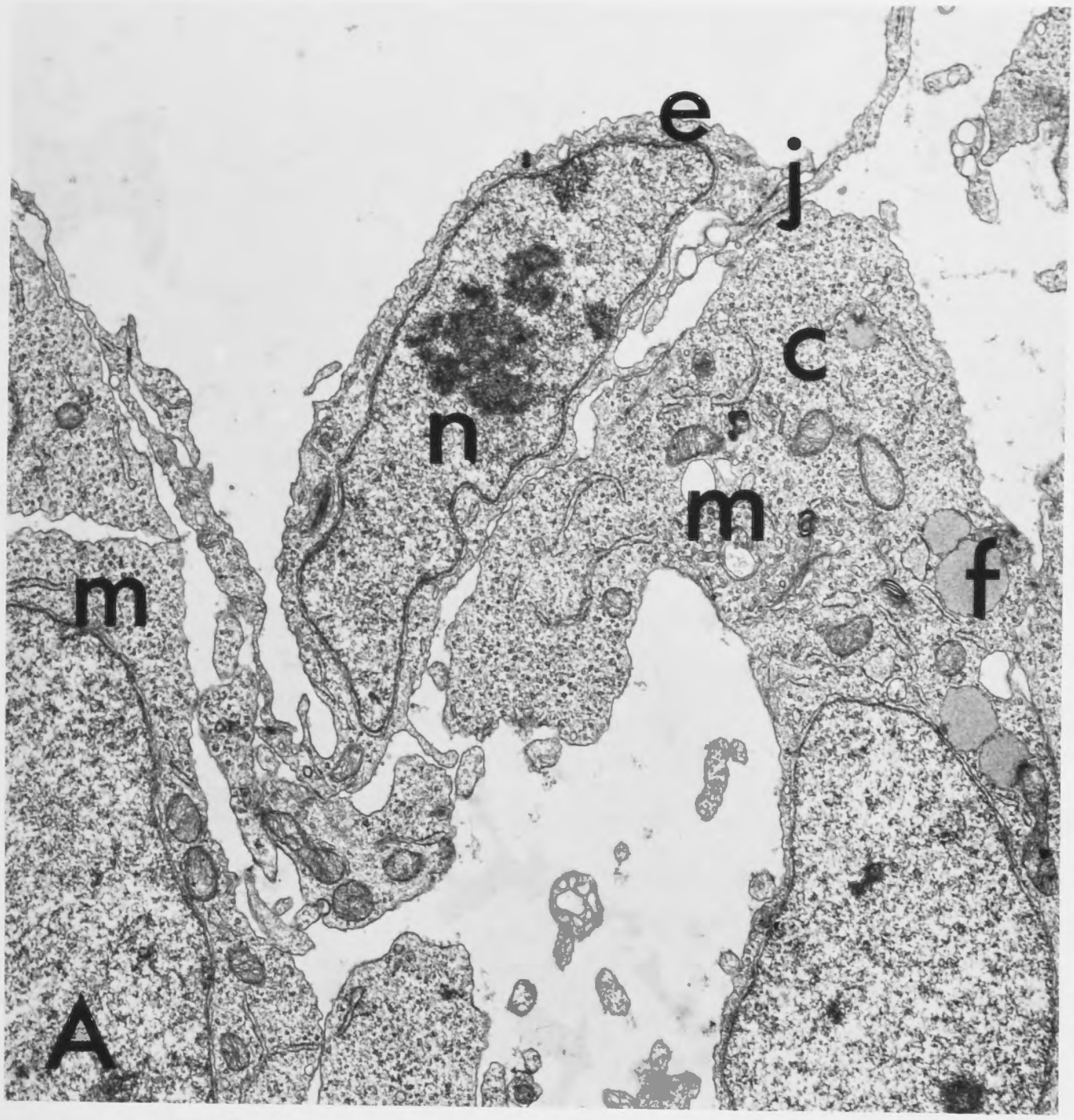
Fig. 4.5A Electron micrograph from the middle digit of the hind limb of a normal 6 day old CC chick embryo to show the structure of a blood vessel. The endothelial cells (e) of this vessel contain a large oval nucleus (n). A junction between two adjacent endothelial cells is indicated (j). The underlying mesenchymal cells (m) have a cytoplasm (c) containing many ribosomes and short profiles of endoplasmic reticulum. Fat droplets (f) are also seen.

Magnification 14,000 x.

Fig. 4.5B Electron micrograph to show a vessel in the digit of a normal 10 day chick embryo. The endothelial cells (e) which contain fat droplets (f) are surrounded by closely apposed periendothelial cells (p). The surrounding tissue space contains scattered collagen fibres (cf) and elongate mesenchymal cells (m).

Magnification 10,000 x.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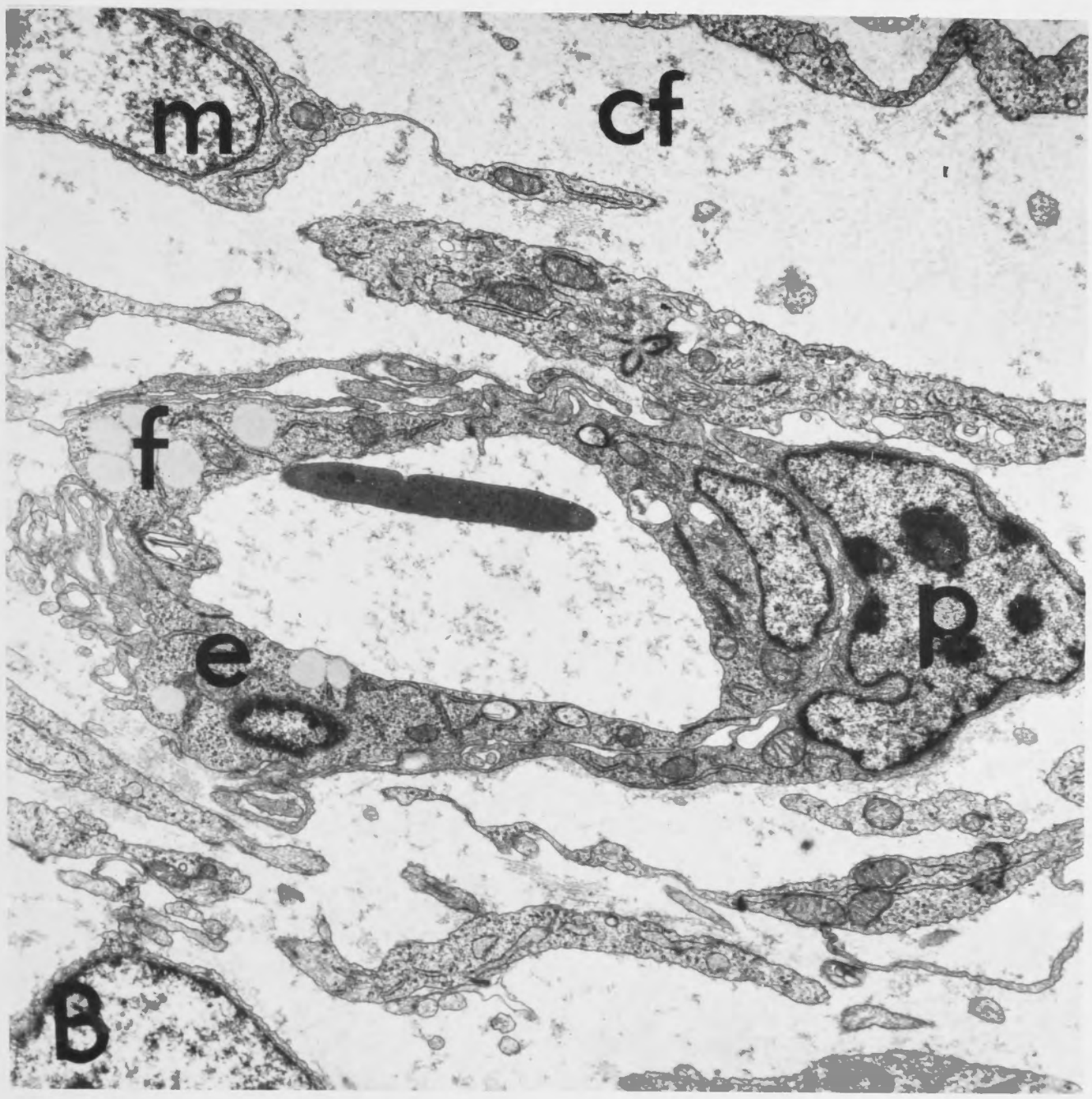
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cells but, in most vessels, there was no basement membrane, although accumulations of electron dense material were occasionally seen under the endothelial layer at this stage. Collagen fibres are now seen throughout the intercellular space, either singly or in small bundles. The mesenchymal cells have an elongate or stellate appearance.

A similar vessel is shown in fig. 4.6A and illustrates some of the cytoplasmic organelles within the endothelial cells at this stage; these include profiles of rough endoplasmic reticulum, round or tubular mitochondria and larger vacuoles, a few small pinocytotic vesicles and numerous ribosomes. The periendothelial cells are similar in appearance.

Adjacent endothelial cells in these vessels may make contact over a very short region (fig. 4.6A) or may interdigitate by long overlapping cell processes (fig. 4.6B). There may also be relatively large areas of apposition between two adjacent endothelial cells (fig. 4.6C).

The wall of a vessel from a 12 day normal CC embryo is shown in fig. 4.6D. By this stage, some of the vessel walls have become more complex. Accumulations of electron dense material can be seen directly beneath the endothelium which indicates the development of a basement membrane. The vessel wall is strengthened by the presence of many overlapping and interdigitating cell processes which derive both from endothelial and periendothelial cells. The number of collagen fibres has also increased.

Blood vessels in the hind limb of embryos inoculated with allogeneic or syngeneic blood - light microscopy

A transverse section of the middle digit from the hind leg of a 12 day old AA embryo inoculated at 6 days with syngeneic adult blood is shown in fig. 4.7A. The vessels appear normal and there are no extravasated red blood cells within the tissues.

Fig. 4.7B, 4.7C and fig. 4.8 are sections from the middle digit of the hind leg of CC embryos inoculated on the CAM at 6 days with diluted adult AA blood. As mentioned earlier in this chapter, this procedure results in widespread haemorrhage. The number of haemorrhages in the digit appeared to increase with time after inoculation, although

Fig. 4.6A Detail of the wall of a vessel in the digit of a normal 10 day old CC chick embryo. A periendothelial cell (p) is closely apposed to the underlying endothelial cells (e) which abut to form a short junctional area (j). A tubular shaped mitochondrion is also marked (mt). The vascular lumen contains a vacuolated (v) haematogenous cell (h).

Magnification 25,000 x.

Fig. 4.6B Detail to show the junctional area (j) between two adjacent endothelial cells (e) in a vessel from the digit of a normal 10 day CC embryo. The area of contact between the two endothelial cells is increased by the presence of long overlapping processes (o). A periendothelial cell (p) is closely apposed to the endothelium.

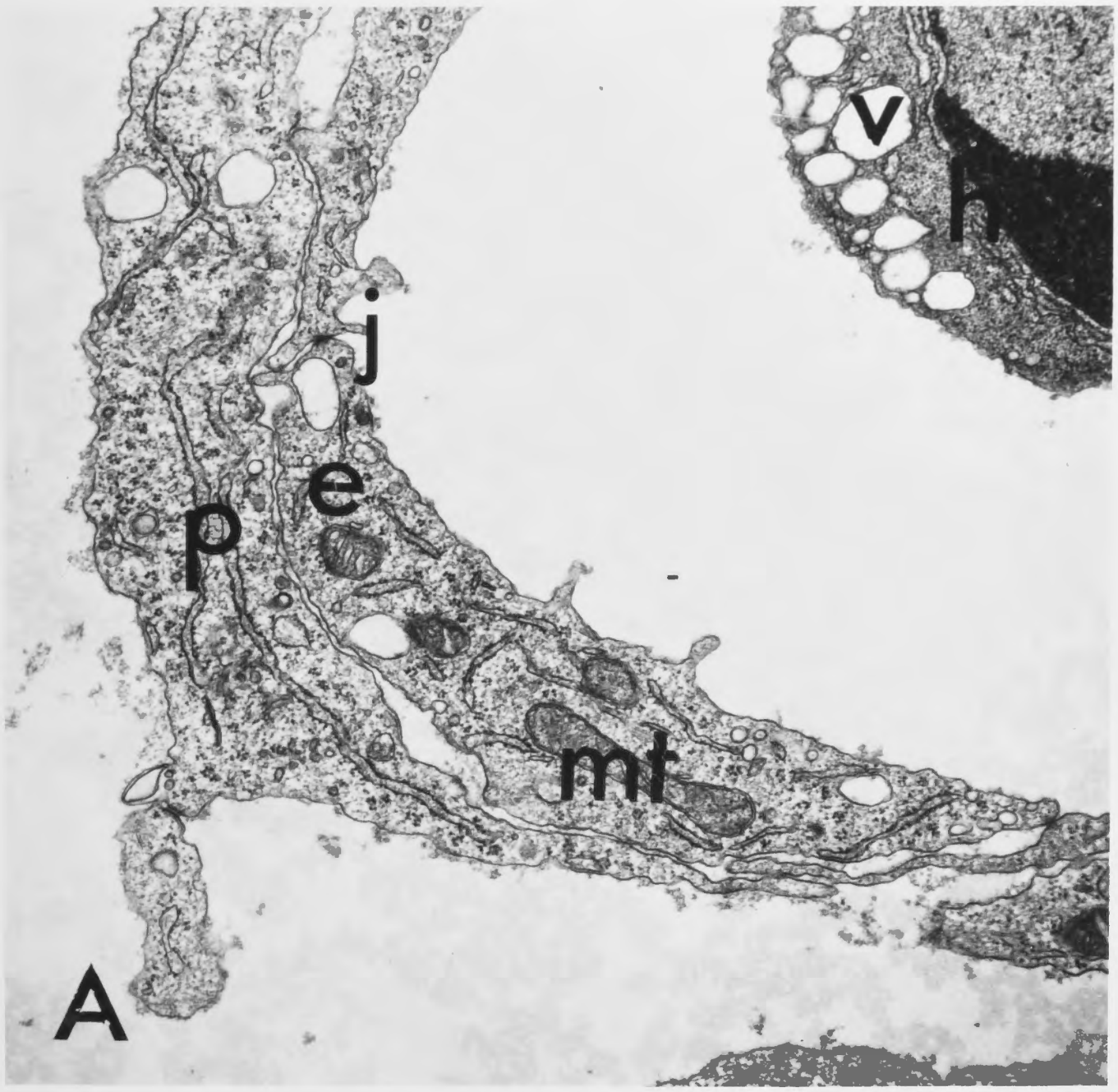
Magnification 23,000 x.

Fig. 4.6C Another endothelial junction (j) in a vessel from the digit of a normal 10 day CC chick embryo. The endothelium (e) and the vessel lumen (l) are also marked.

Magnification 23,000 x.

Fig. 4.6D Vessel wall from a vessel in the digit of a normal 12 day embryo. Mesenchymal cells (m) and the overlapping processes of periendothelial and endothelial cells (op) are present under the endothelial layer (e). The vessel lumen (l) is also marked.

Magnification 9,400 x.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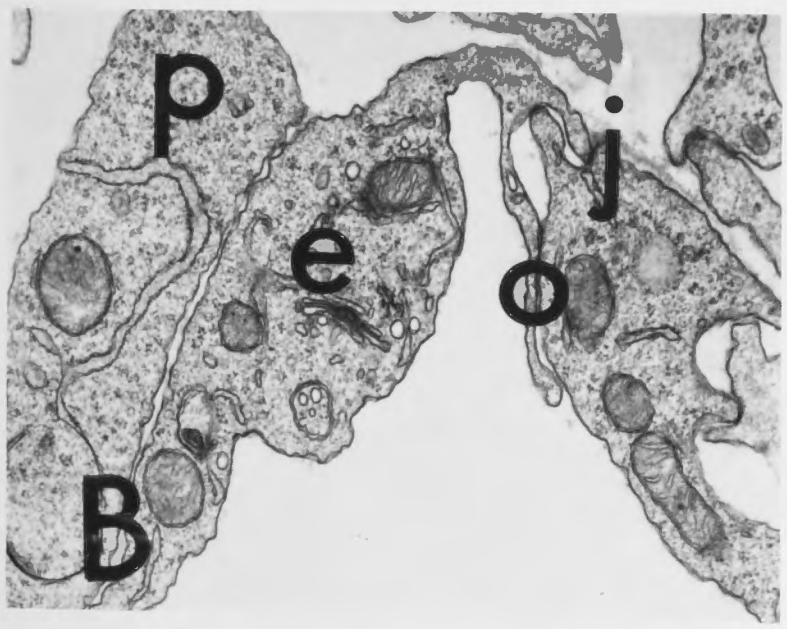
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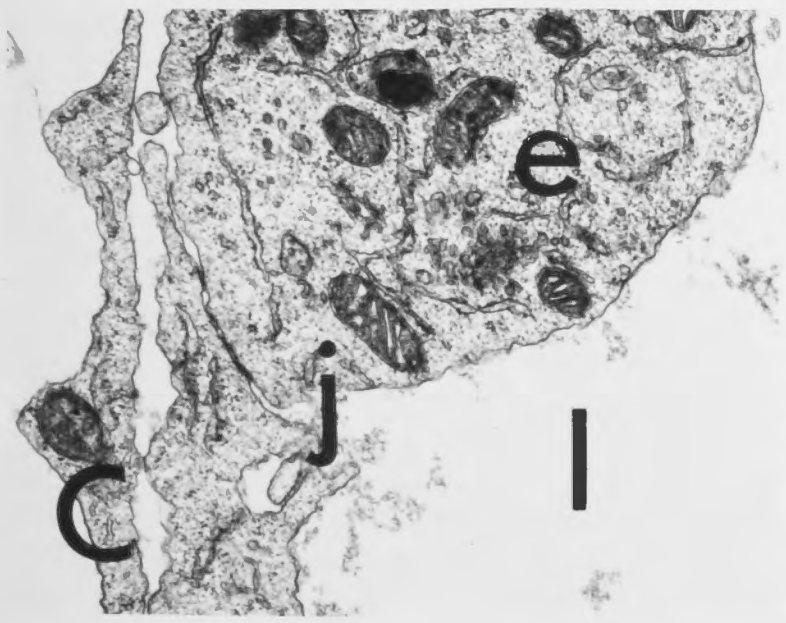
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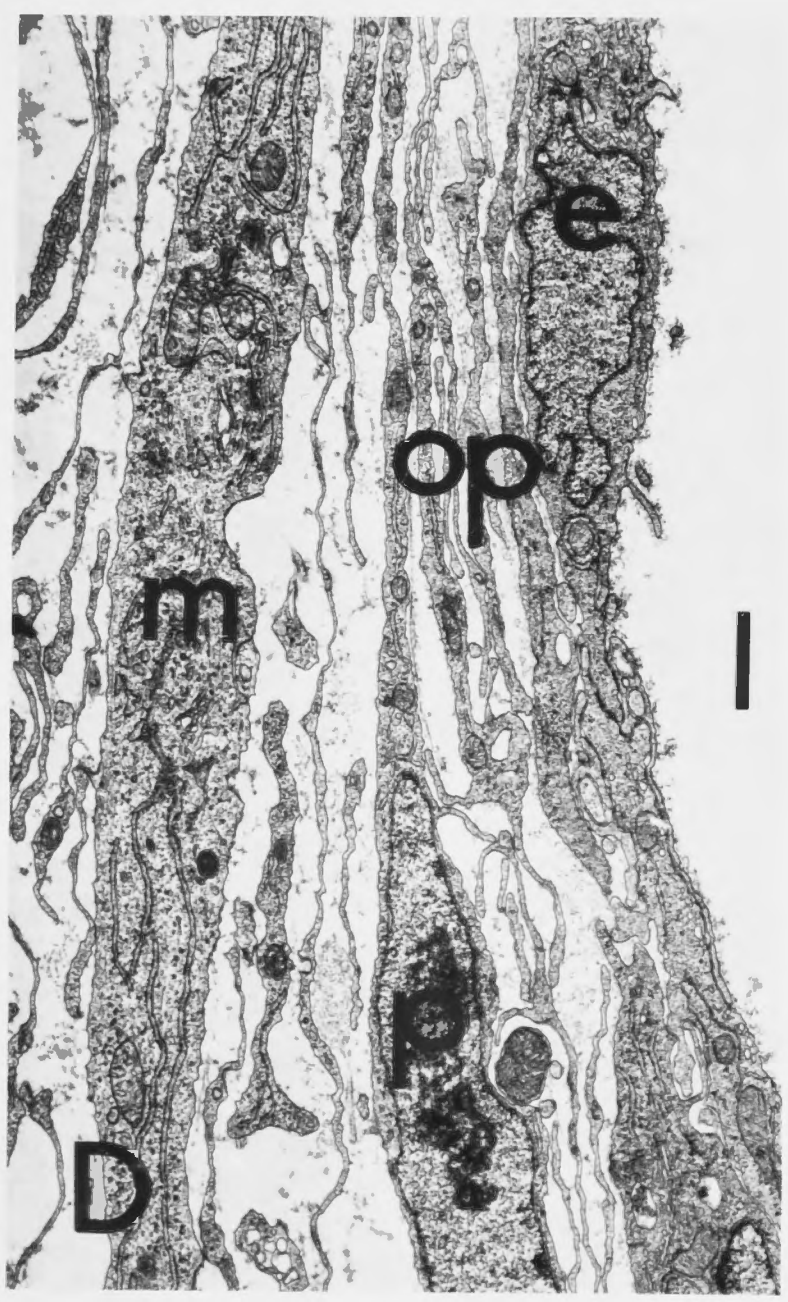
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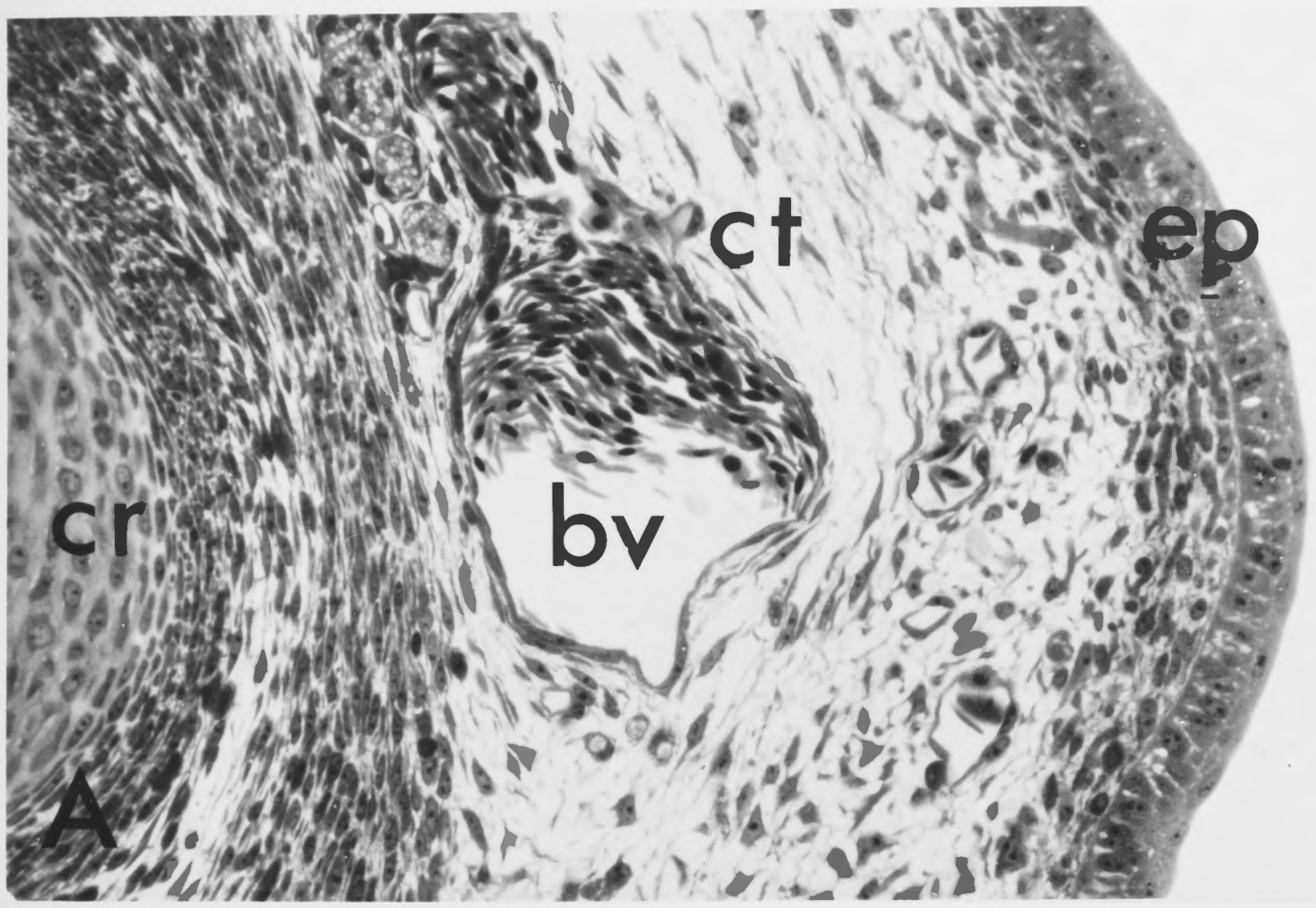
Fig. 4.7A Transverse section from the digit of a 12 day AA embryo inoculated on the CAM at 6 days with adult syngeneic blood. The blood vessels (bv), connective tissue layer (ct), epidermis (ep) and central cartilage (cr) are all normal in appearance.
Azure II/methylene blue Magnification 400 x.

Fig. 4.7B Transverse section from the digit of an 8 day CC chick embryo, 2 days after the inoculation of adult allogeneic blood on the CAM. Extravasated red blood cells (e) appear in the tissue spaces, but the surrounding connective tissue (ct) and muscle bundles (m) are normal.
Azure II/methylene blue Magnification 275 x.

Fig. 4.7C Transverse section through the digit of a 9 day CC embryo to show the development of haemorrhagic lesions 3 days after the inoculation of allogeneic blood. A red blood cell (b) is escaping through a gap in a vessel wall and extravasated red blood cells (e) accumulate in the surrounding tissue spaces. A large gap (g) has developed in an adjacent vessel.
Azure II/methylene blue Magnification 400 x.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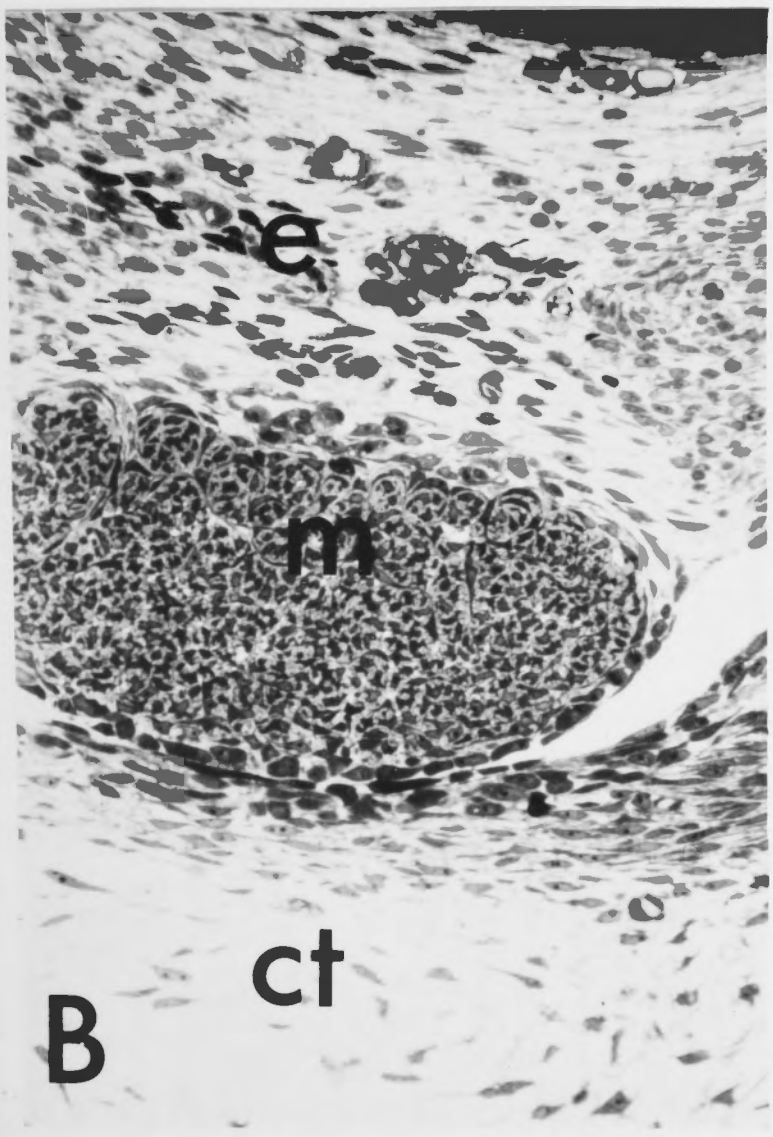
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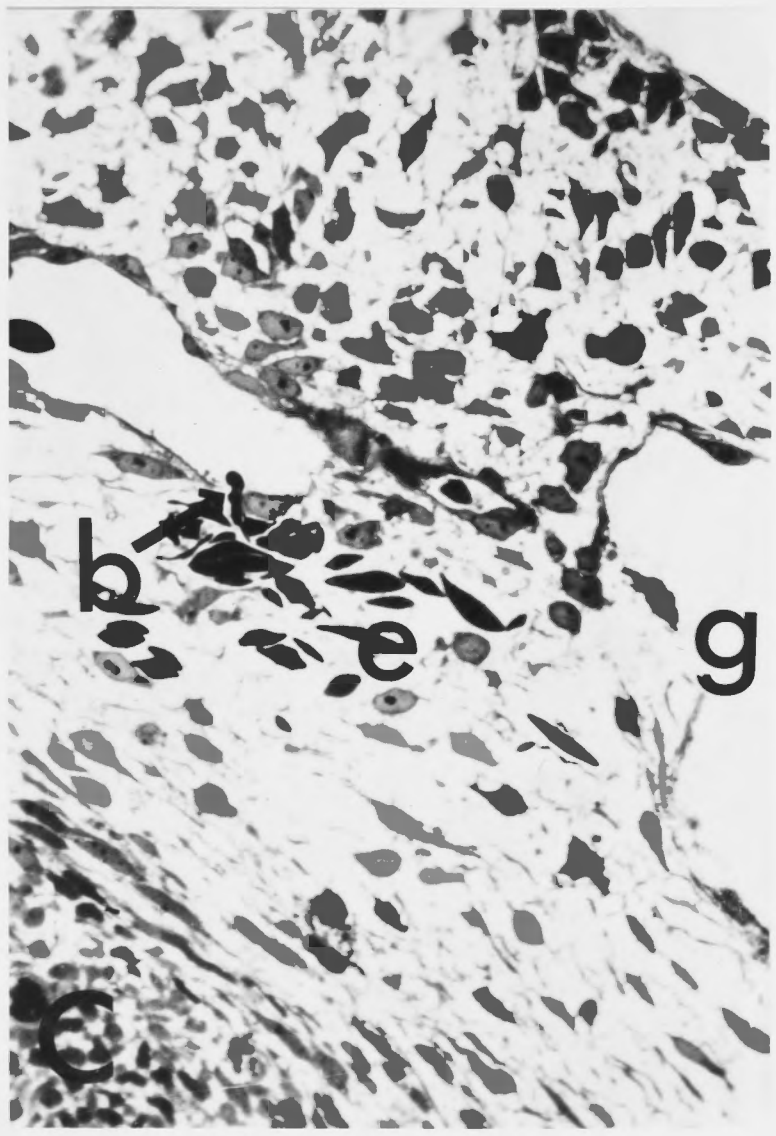
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they took at least 2 days to develop.

Fig. 4.7B illustrates the haemorrhagic lesion in an 8 day CC embryo, 2 days after inoculation. Although accumulations of extravasated red blood cells are seen in the tissues, frank gaps could not be detected in the lining of vessels. Other tissues of the digit remained normal in appearance.

Fig. 4.7C shows a transverse section 3 days after inoculation. Red cells may be seen escaping into the surrounding tissues through gaps in the vessel walls; in some areas the integrity of the endothelium is completely disrupted. No degenerative changes were seen in extravasated red blood cells and other tissues of the digit appeared normal.

Four days after the inoculation of diluted AA blood (fig. 4.8A, B, C) haemorrhages are very common, large numbers of red cells accumulate in the tissue spaces (fig. 4.8A) and the vessels are frequently congested with red cells (fig. 4.8B). In many areas the tissue spaces are crowded with degenerating red cells.

Blood vessels in the hind limb of embryos with haemorrhagic lesions - electron microscopy

Inbred CC embryos, inoculated on the CAM at 6 days of age with diluted adult AA blood were sampled for electron microscopy at day 10, 4 days after inoculation. Some recipients had extensive areas of haemorrhage, while in others only a few scattered petechial haemorrhages were seen on the body surface. The electron microscopic appearance of vessels in the digit of the hind limb of these recipients showed a correspondingly wide range of damage.

In many vessels which otherwise appeared normal, 'blebs' and also long thin cytoplasmic processes of endothelial cells projected into the lumen. These processes usually contained no cell organelles and resembled pseudopodia in other cell types. This is illustrated in figs. 4.9A and 4.9C.

In other vessels, the endothelial cells had a more rounded shape than was seen in normal vessels (compare fig. 4.5B). The endothelial cells protruded into the vessel lumen and appeared lifted off the underlying

Fig. 4.8A-C Transverse sections through the middle digit from the hind limb of a 10 day CC embryo to show the development of haemorrhagic lesions 4 days after the inoculation of diluted adult allogeneic blood on the CAM. Glycol methacrylate sections stained with haematoxylin and eosin.

Fig. 4.8A Accumulation of extravasated red blood cells (e) within the tissue spaces of the digit. Surrounding tissues, such as the muscle bundle (m), appear normal.

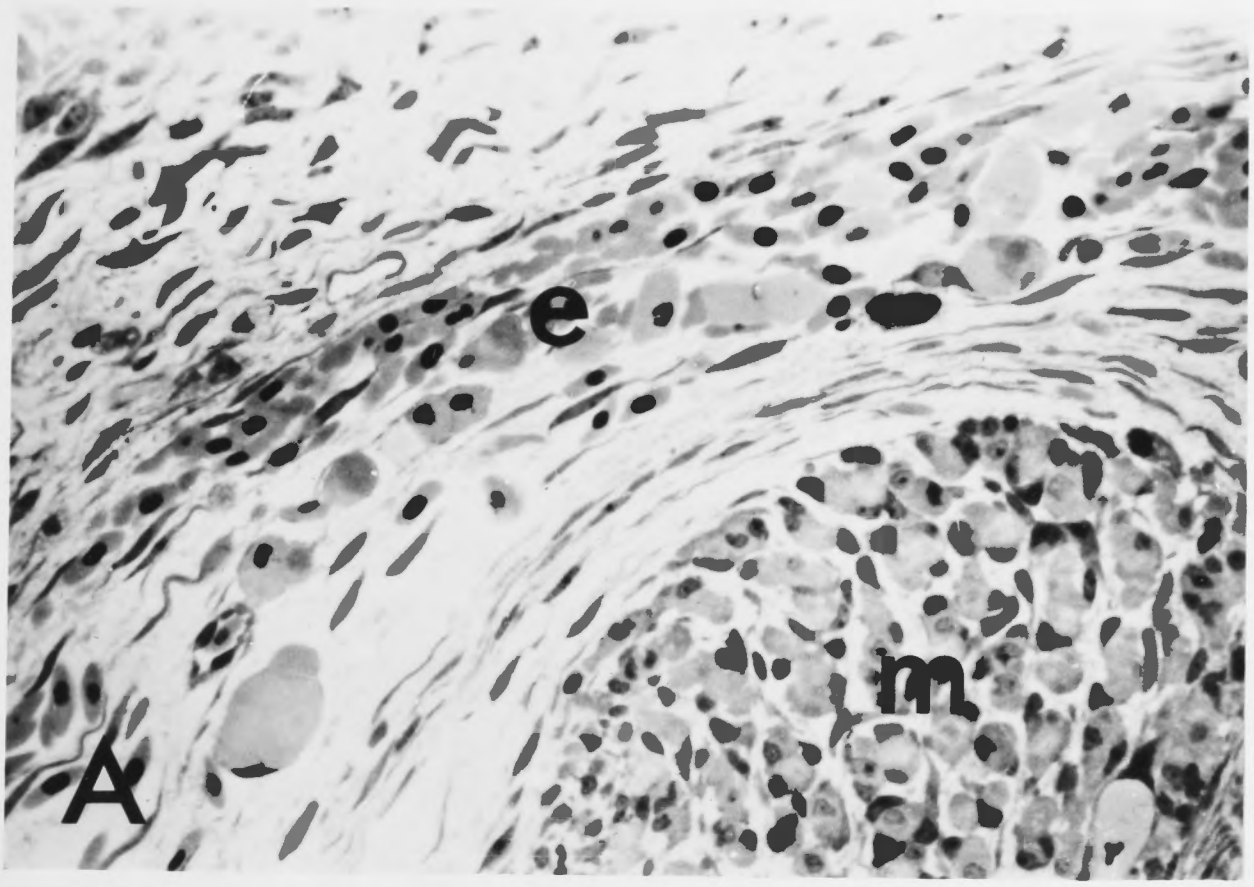
Magnification 325 x.

Fig. 4.8B Vessels in the digit which have become congested with red cells (s). A gap (g) has developed in one of the vessel walls and extravasated red blood cells (e) accumulate in the tissue spaces.

Magnification 325 x.

Fig. 4.8C Accumulation of degenerating red blood cells (d) within the connective tissue layer.

Magnification 325 x.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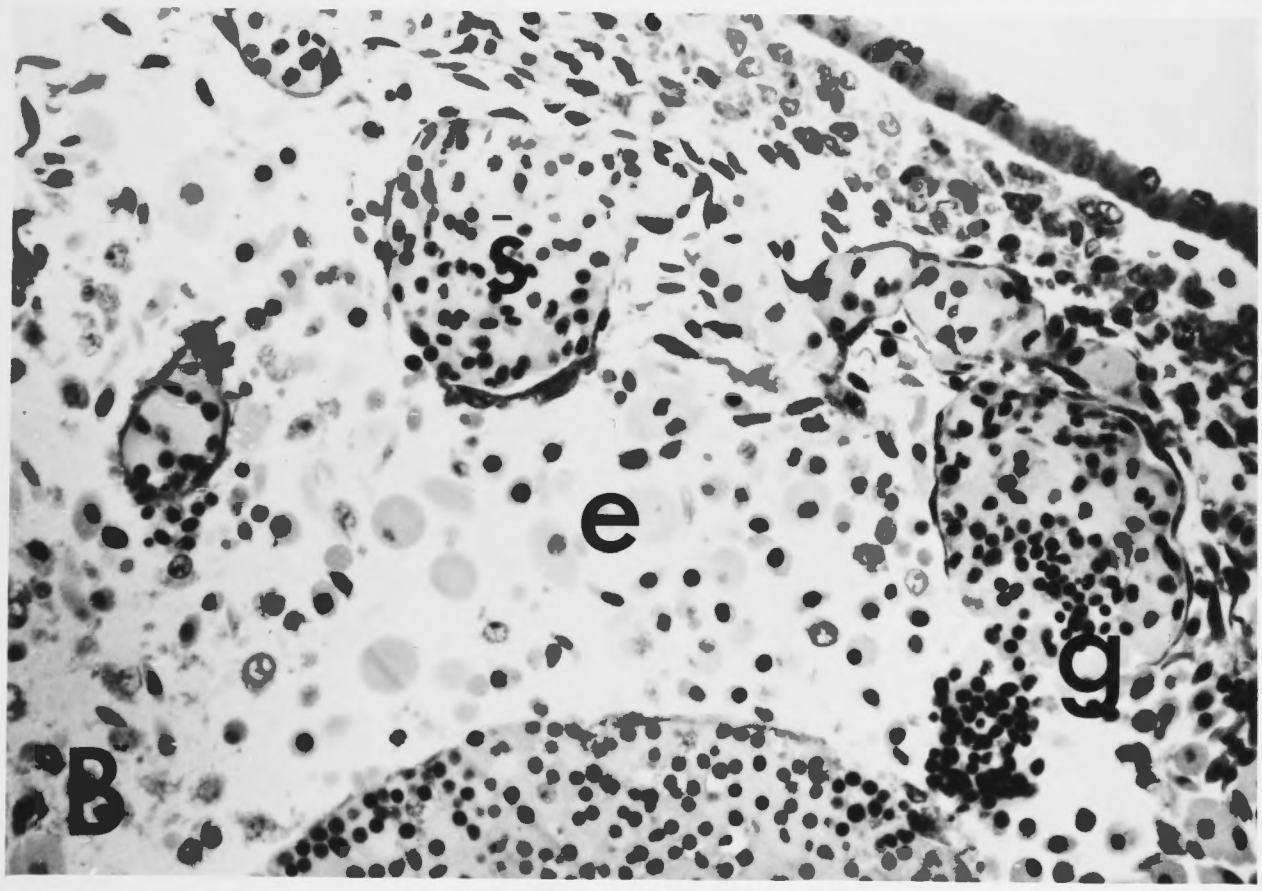
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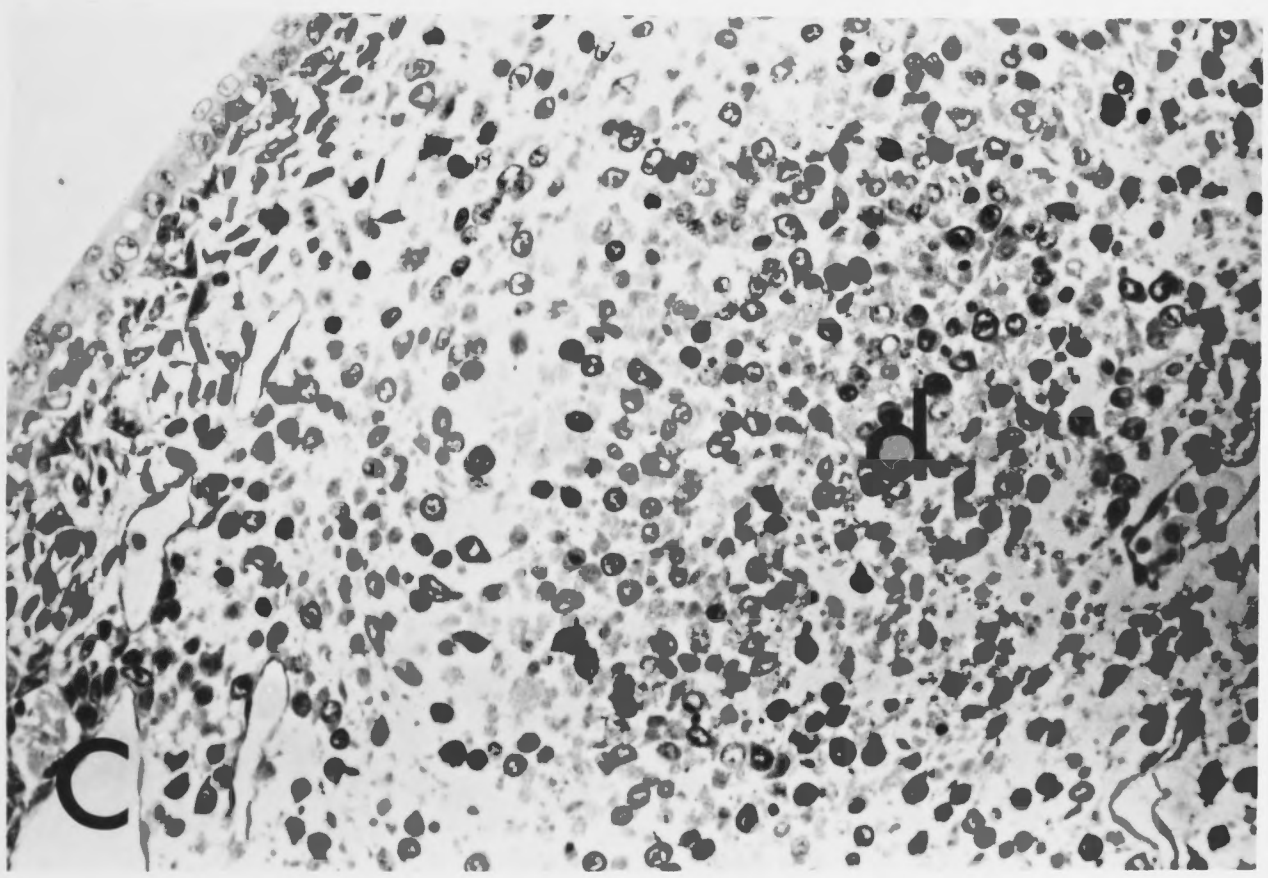
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Fig. 4.9A-D Electron micrographs to show the development of 'blebs', extrusion of cytoplasmic processes and other changes which occur in the vessels of the digit of a 10 day CC chick embryo, 4 days after the inoculation of diluted adult AA blood onto the CAM.

Fig. 4.9A The development of cytoplasmic 'blebs' (b) from an endothelial cell (e) which otherwise appears normal is shown in this micrograph. Surrounding periendothelial cells (p) and mesenchymal cells (m) also appear normal.

Magnification 10,000 x.

Fig. 4.9B Endothelial cells with an abnormally rounded shape (e). An electron dense inclusion (i) is seen in one of these cells. The surrounding periendothelial cells (p) appear normal.

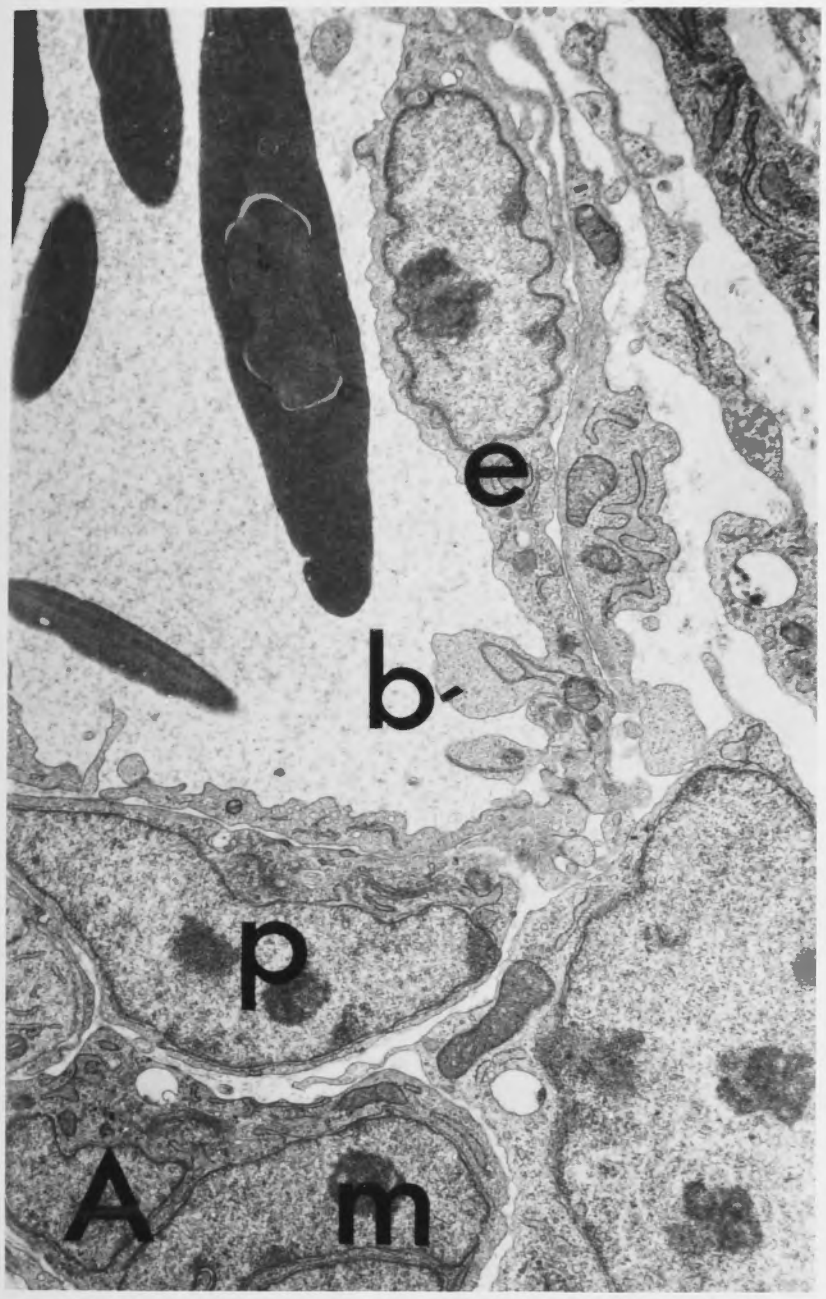
Magnification 25,000 x.

Fig. 4.9C A vessel in which an endothelial cell (e) extrudes long cytoplasmic processes (pr) into the vascular lumen.

Magnification 14,000 x.

Fig. 4.9D In this vessel, one endothelial cell has an abnormally dense cytoplasm (c) containing dilated sacs of endoplasmic reticulum (d) and fat droplets (f). Adjacent endothelial cells (e) and periendothelial cells (p) appear normal.

Magnification 24,000 x.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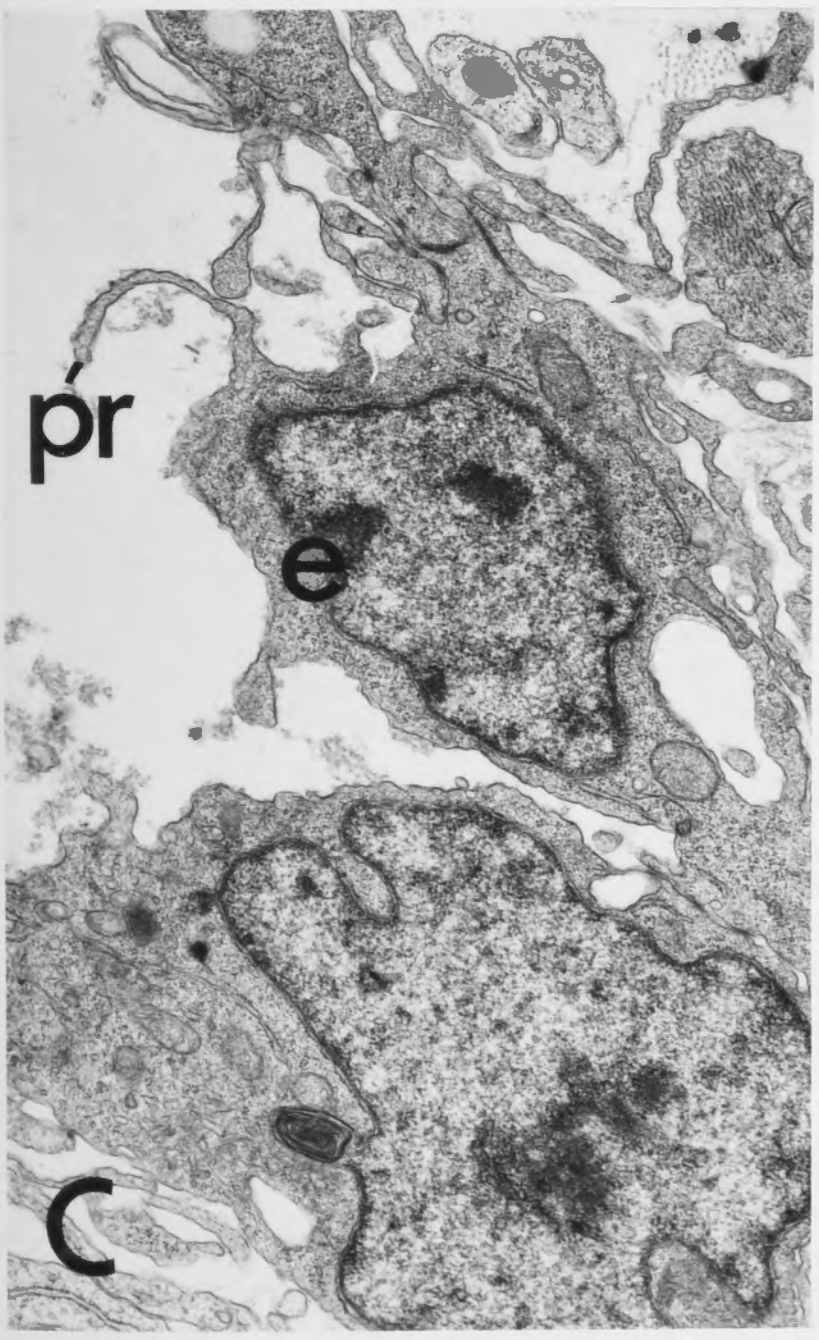
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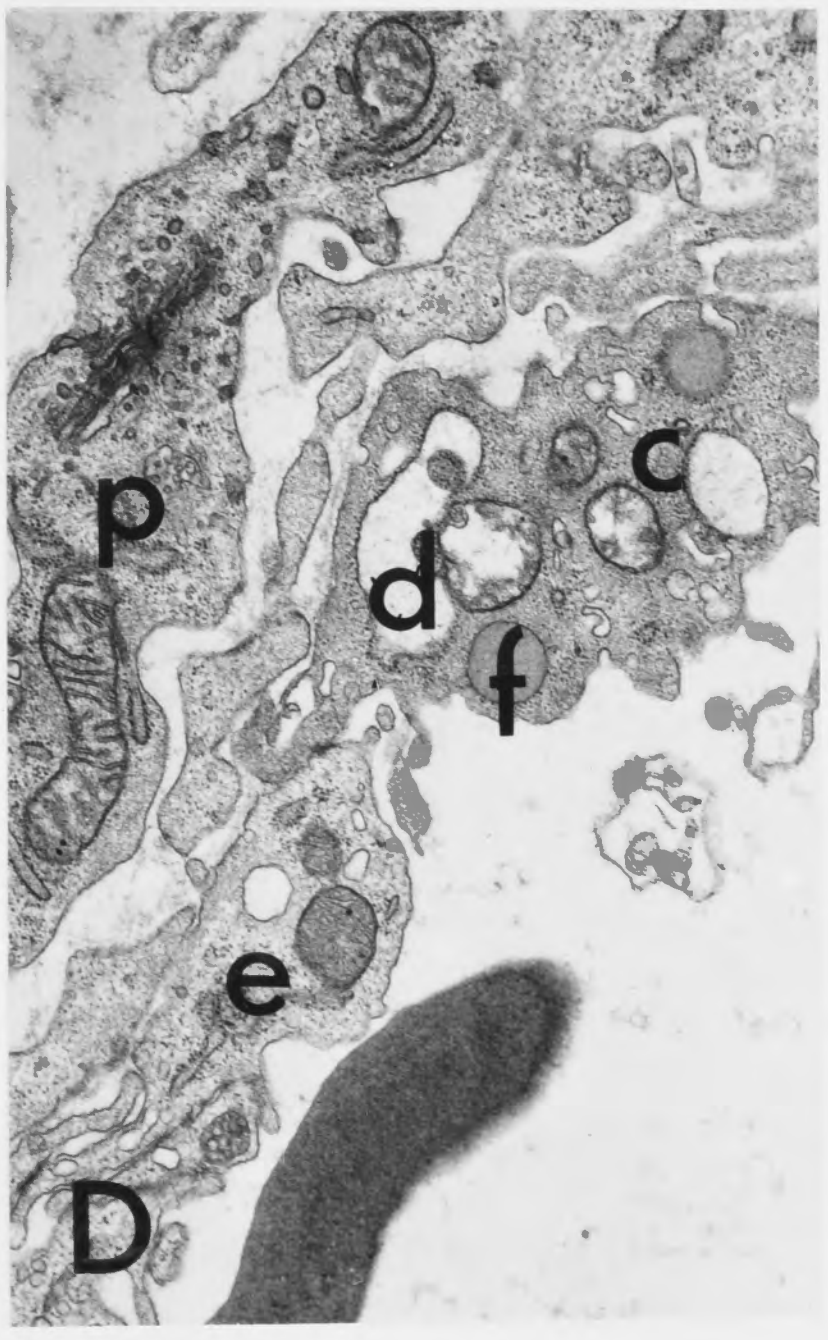
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periendothelial cells. A vessel in which changes of this kind have occurred is shown in fig. 4.9B. This figure also shows the type of inclusions sometimes seen in these endothelial cells. They contain electron dense material which resembles autolysing cellular components.

Some endothelial cells had a denser cytoplasmic matrix and contained dilated sacs of endoplasmic reticulum and fat droplets, while adjacent endothelial cells were often normal in appearance (fig. 4.9D). Damage of this kind appeared to lead to focal degeneration of the endothelium and eventually to frank gaps. Fig. 4.10A shows such a vessel with only the 'ghost' of an endothelial cell remaining. Adjacent endothelial and periendothelial cells appear unchanged.

In embryos with severe haemorrhages, gaps in the endothelium were frequently seen in the vessels of the digits. A typical example of such a gap is shown in fig. 4.10B where red blood cells can be seen escaping into the surrounding tissue spaces. Degenerative changes in extravasated red blood cells were rare and mesenchymal cells in these areas appeared normal. In some instances, vessels were seen in which the entire endothelium had almost disintegrated. Only membranous material and some cell nuclei remained to show the original position of the vessel (fig. 4.10C).

Blood vessels in the CAM of embryos with haemorrhagic lesions - light microscopy

As mentioned earlier, the inoculation of adult allogeneic blood into 6 day embryos also causes petechial haemorrhages to develop in the CAM. In this tissue the blood vessels are well developed (Sethi and Brookes, 1971) in contrast to the rather primitive appearance of those in the digit. Blood vessels in the CAM of these diseased embryos were therefore examined by light and electron microscopy to determine whether similar changes occurred.

Fig. 4.11 shows a section through the CAM of a 12 day CC embryo, 6 days after the inoculation of 10^6 adult AA leukocytes. A number of smaller, thin walled blood vessels are congested and extravasated red blood cells have accumulated within the mesenchymal layer. Larger vessels, such as the artery shown in fig. 4.11, are well different-

Fig. 4.10A-C Electron micrographs to show the development of gaps in the wall of vessels in the digit from a 10 day CC embryo, 4 days after the inoculation of diluted adult AA blood on the CAM.

Fig. 4.10A The 'ghost' of a degenerated endothelial cell (d) in a vessel in which the surrounding endothelial cells (e) and periendothelial cells (p) are normal in appearance.

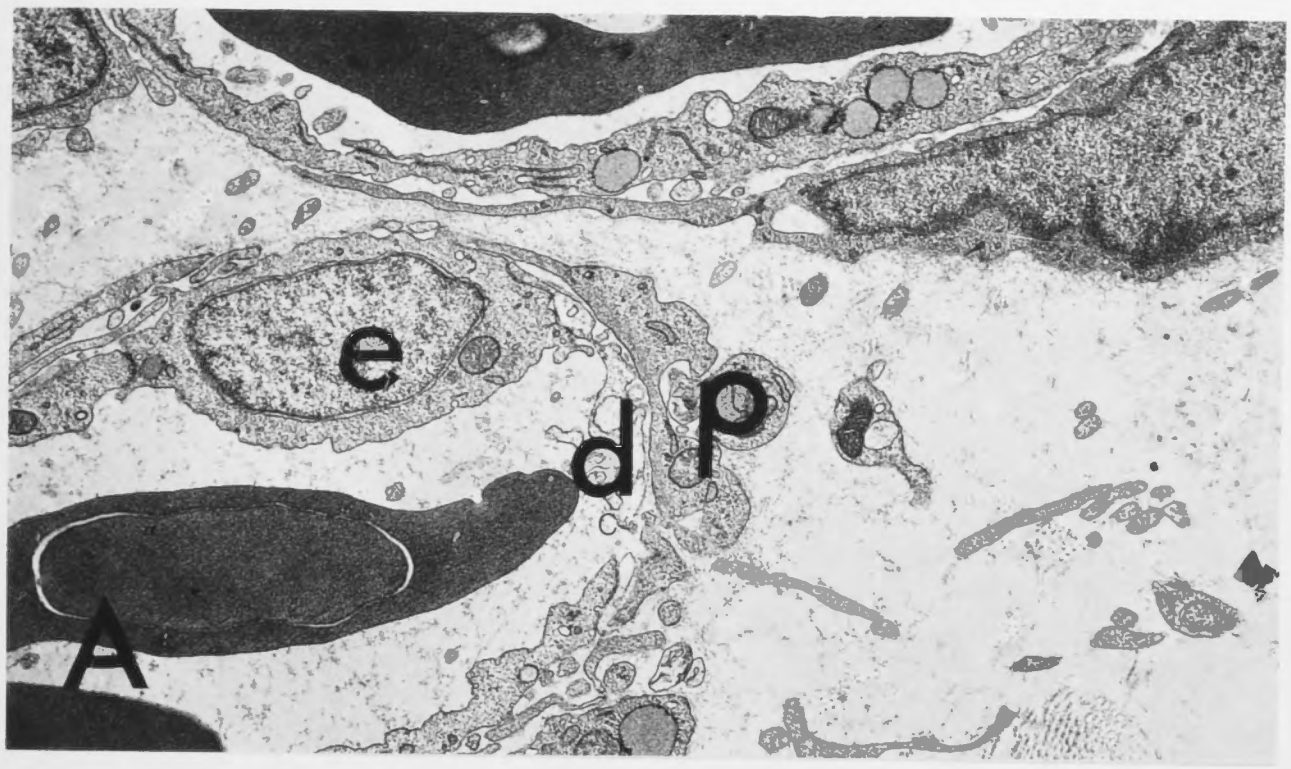
Magnification 10,000 x.

Fig. 4.10B Extravasation of red blood cells (er) from the vascular lumen (l) into the surrounding tissue space through a gap (g) which has developed between two adjacent endothelial cells (e). A mesenchymal cell (m) is also marked.

Magnification 10,000 x.

Fig. 4.10C A degenerating epithelial cell (d) from a severely damaged vessel. The vessel lumen is marked (l).

Magnification 9,400 x.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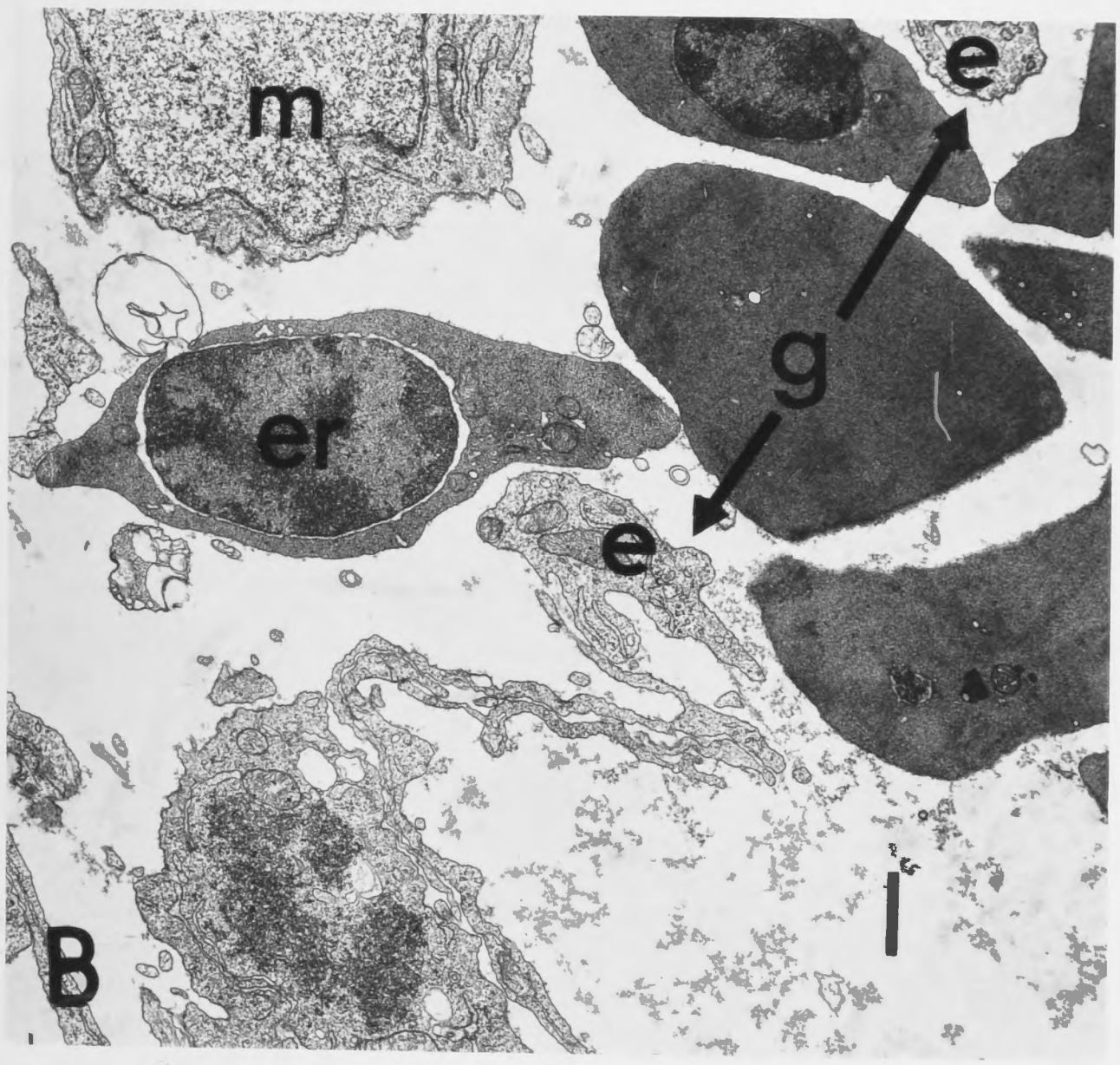
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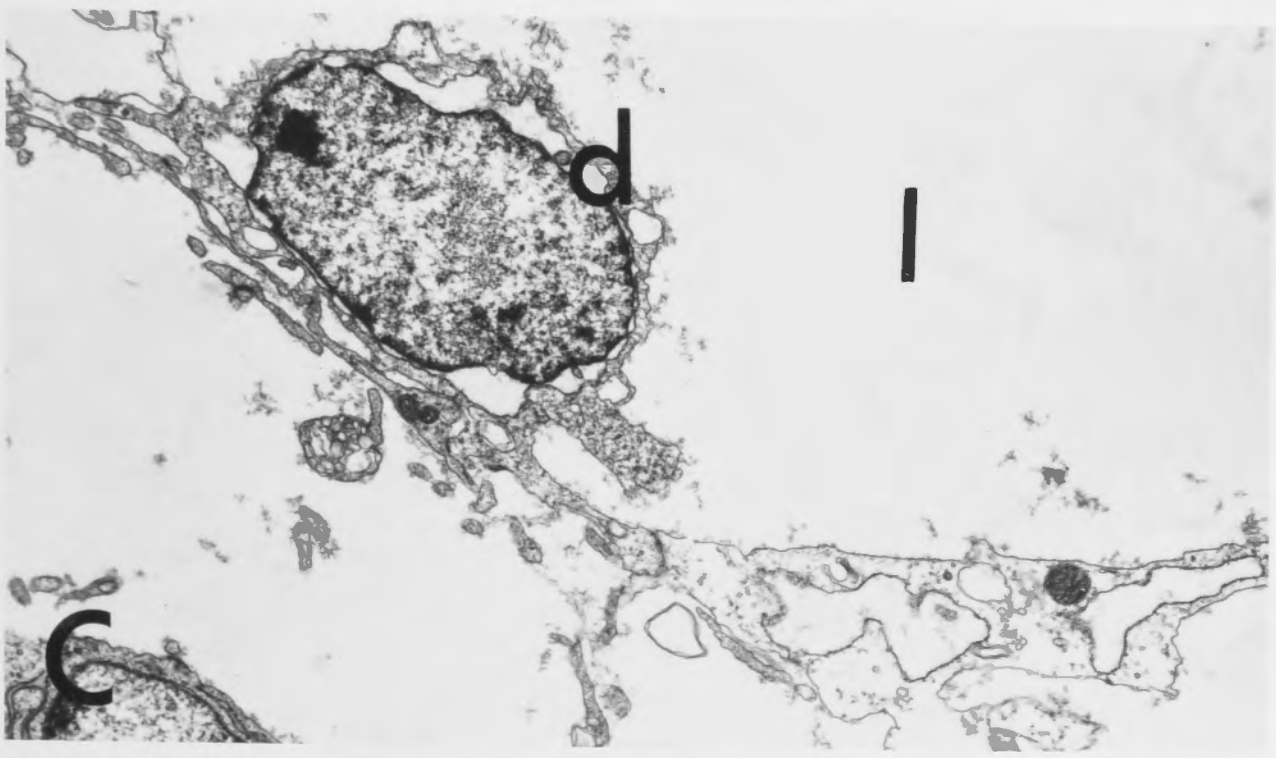
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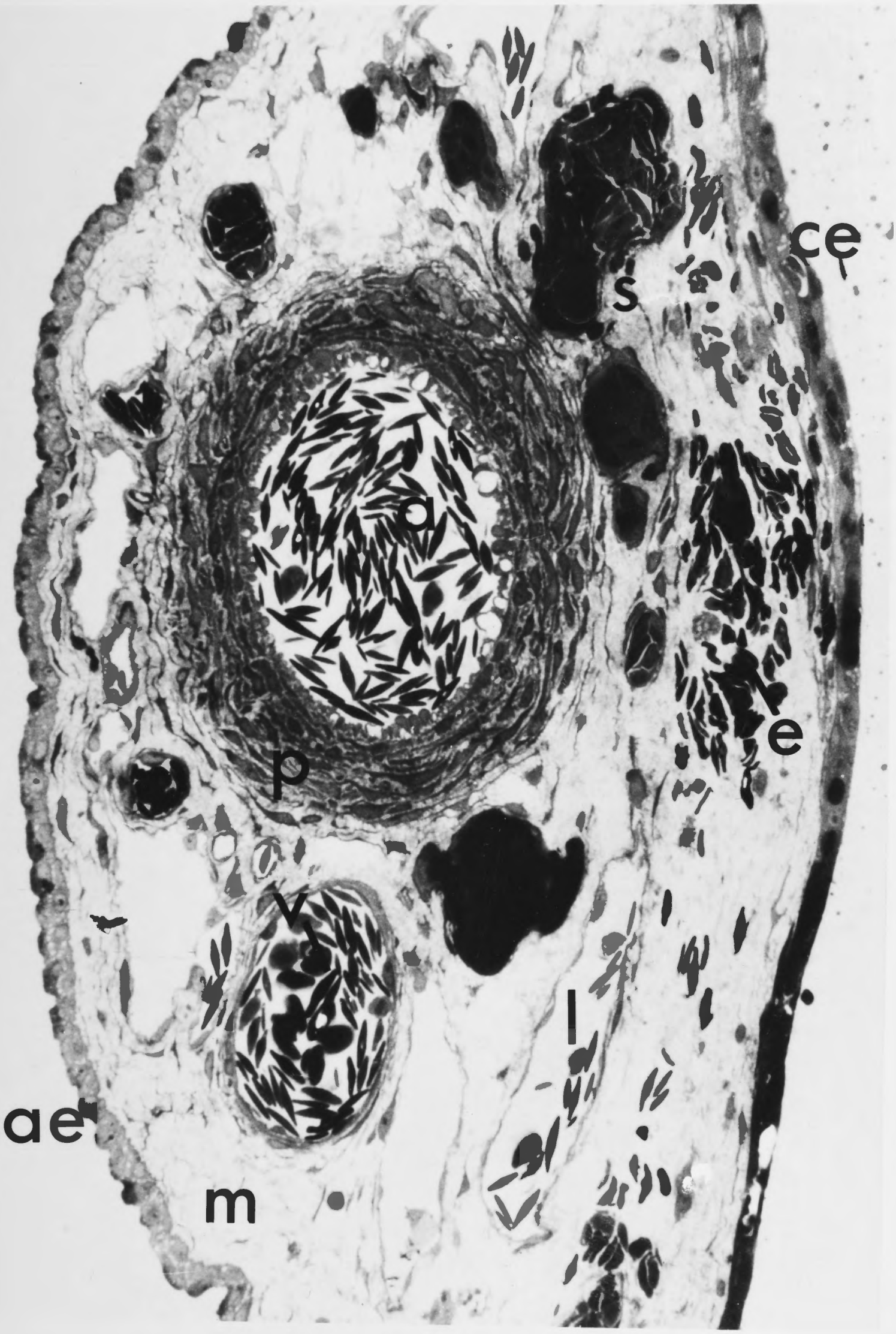
Fig. 4.11 Section from the CAM of a 12 day CC embryo which had been inoculated at 6 days with 10^6 adult AA leukocytes to show the changes which occur in the CAM of embryos with haemorrhagic lesions. The large arterial vessel (a) which is invested with a thick sheath (p) appears undamaged. Some small thin-walled vessels (l and v) appear normal, while others (s) are congested with red blood cells. The mesenchymal layer (m) around these vessels contains accumulations of extravasated red blood cells. The chorionic epithelium (ce) and allantoic epithelium are also marked.
Azure II/methylene blue Magnification 575 x.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iated and have acquired a thick sheath. No distinct lesions were seen in these vessels.

Blood vessels in the CAM of embryos with haemorrhagic lesions - electron microscopy

At the level of the electron microscope, congested vessels such as shown in figs. 4.11 and 4.12A were lined by attenuated endothelial cells which often were invested with at least a discontinuous basement membrane and closely applied periendothelial cells. Frequently no abnormal changes, other than intravascular packing of red cells, could be detected.

Vessels assumed to be arterial, did not show any degenerative changes (fig. 4.12B). The crowding of endothelial cells in this particular picture is thought to be the result of vessel constriction either before or possibly during fixation. The endothelial cells otherwise appeared normal.

Most changes were seen in thin walled small vessels of the CAM. A detail of the wall of such a vessel is shown in fig. 4.12C. Although the endothelial cells do not show any abnormalities, two blood-bourne cells seem to adhere to the luminal surface. The relationship of the endothelium to the underlying periendothelium appears undisturbed.

The endothelial cells shown in fig. 4.12D have a rather more abnormal appearance. The cytoplasm contains a large number of vacuoles and vesicles and irregular processes project from the luminal and basal surfaces.

As in the digit, gaps are formed in the endothelial lining of small vessels through which cells from the blood escape into surrounding tissues. Fig. 4.13A shows a red cell escaping through such a gap in the endothelium and in fig. 4.13B a cell, resembling a thrombocyte, appears to pass into the surrounding tissue. As thrombocytes as well as red blood cells escaped from the vessels, small microthrombi were occasionally observed outside the vessels. They were not seen in the digits which were examined at an earlier stage of incubation.

The small capillaries which form an extensive network in the chorionic epithelial layer (Ganote et al., 1964; Sethi and Brookes, 1971) of the CAM were largely unaffected,

Fig. 4.12A-D Electron micrographs of vessels in the CAM of a 12 day CC chick embryo which has been inoculated at 6 days with 10^6 adult AA leukocytes on the CAM. These micrographs show some of the changes which were found in these vessels 6 days after the inoculation of adult allogeneic blood.

Fig. 4.12A Large thin-walled vessel congested with red blood cells (l). The endothelium (e) and periendothelium (p) however, remain undamaged. The surrounding tissue space contains many collagen fibres (cf) and stellate mesenchymal cells (m).

Magnification 10,000 x.

Fig. 4.12B An undamaged arterial vessel showing the protrusion of endothelial cells (e) into the vessel lumen. The vessel wall contains several layers of periendothelial cells (p).

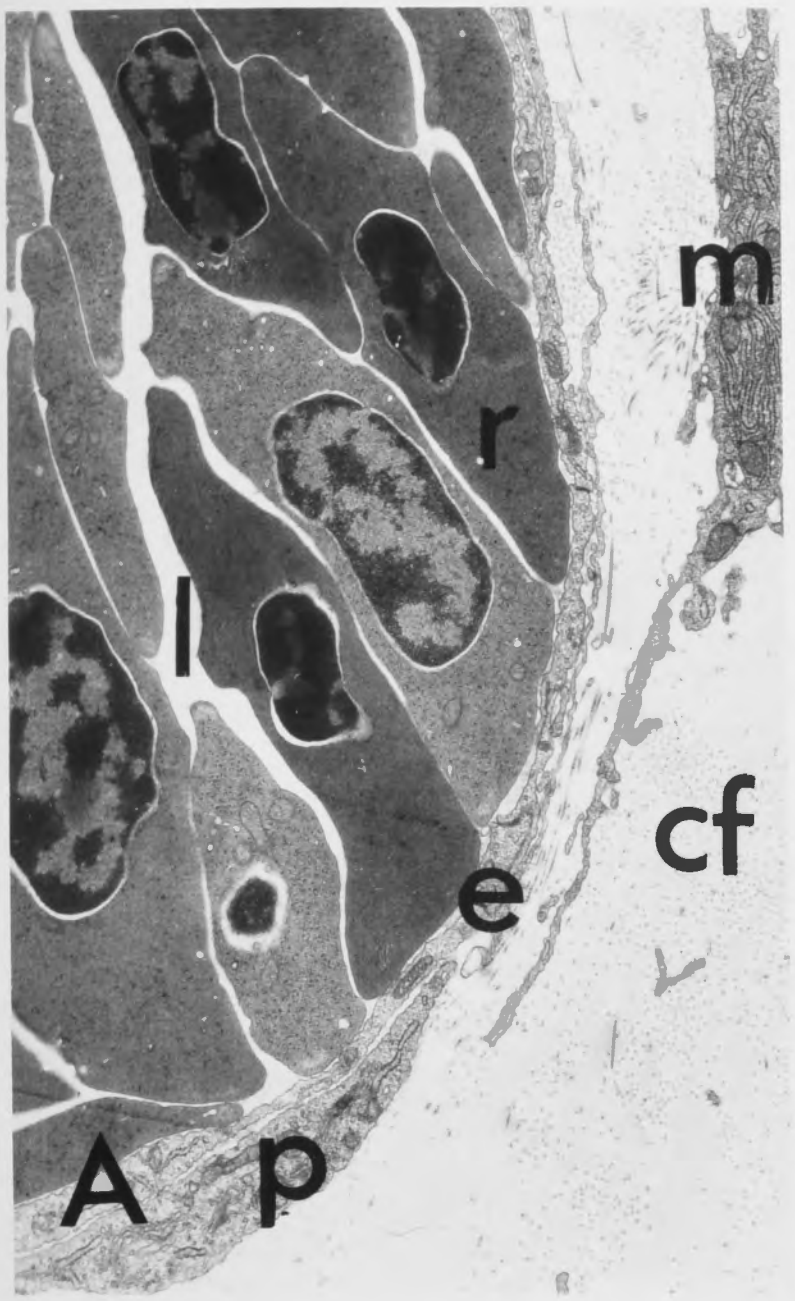
Magnification 10,000 x.

Fig. 4.12C A thin-walled vessel with haematogenous cells (h) closely applied to the luminal surface of the endothelial cells (e). The endothelial cells contain vacuoles (v) and micropinocytotic vesicles indicated by an arrow. The periendothelium (p) is closely apposed to the endothelium and extends small cytoplasmic processes (hk) towards the endothelial cells. A basement membrane (b) is beginning to develop.

Magnification 25,000 x.

Fig. 4.12D A thin-walled vessel in which the endothelial cells (e) contain many vacuoles (v) and micropinocytotic vesicles, indicated by an arrow. Irregular cytoplasmic processes (pr) extend into the vascular lumen. A periendothelial cell is also indicated (pr).

Magnification 25,000 x.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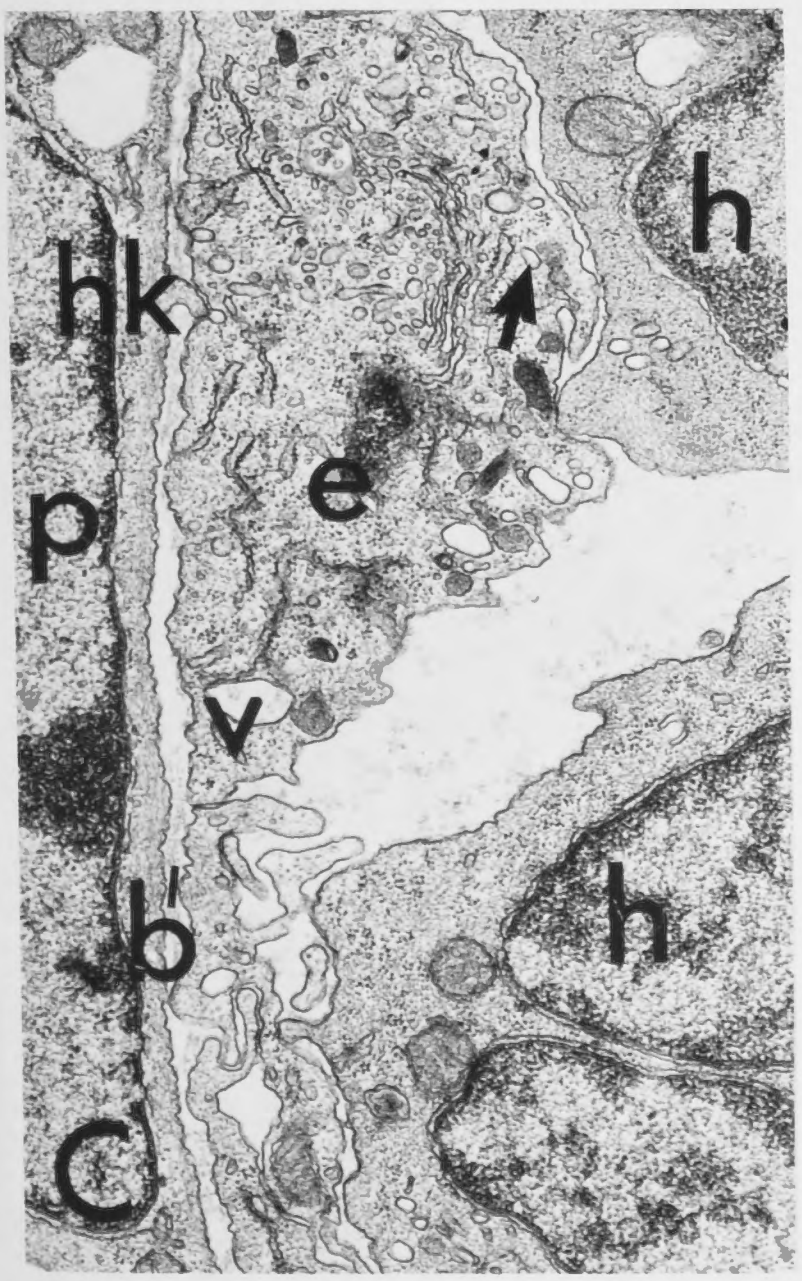
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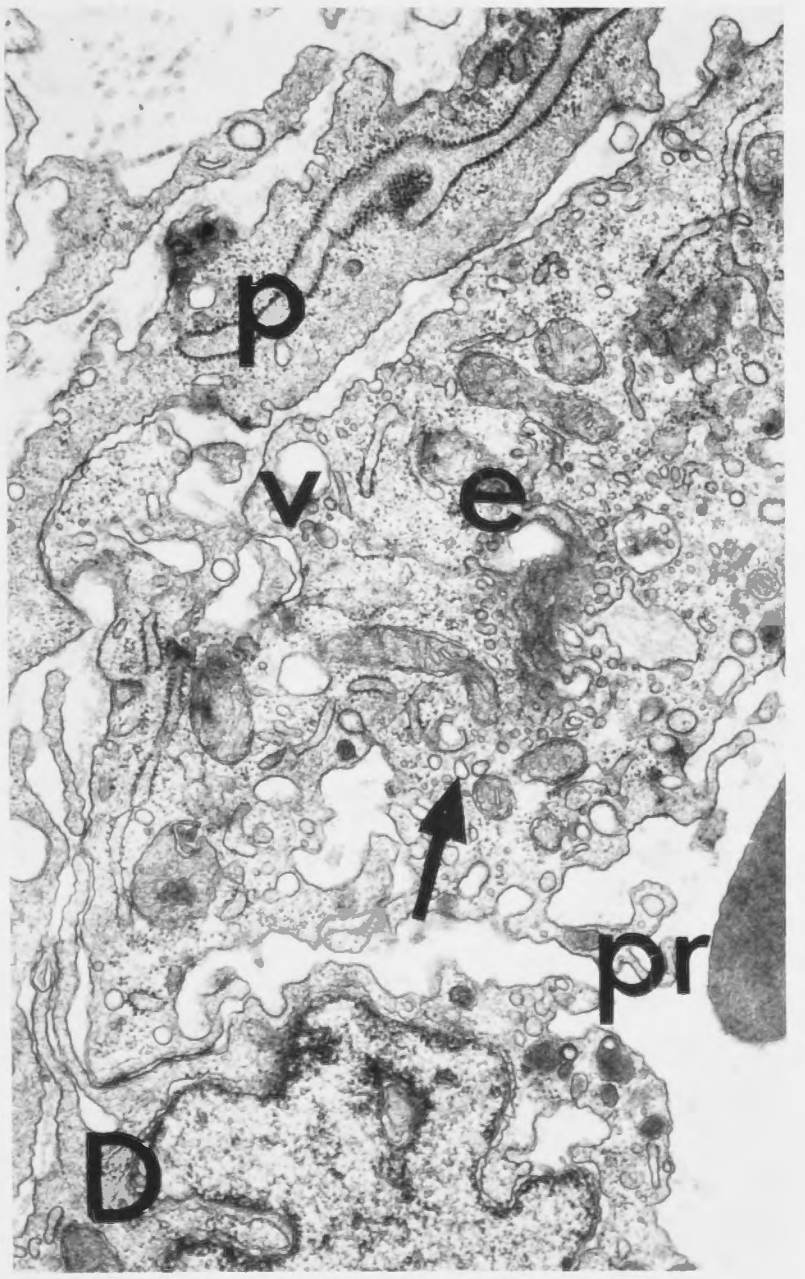
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Fig. 4.13A-D Electron micrograph to show further features of the CAM of a 12 day CC chick embryo which has been inoculated at 6 days with 10^6 adult AA leukocytes.

Fig. 4.13A A red blood cell (er) can be seen escaping through a gap (g) in the endothelium (e) of a thin-walled vessel. A red blood cell within the vessel lumen is also marked (r). The surrounding tissue space contains many collagen fibres (cf). An adjacent vessel (vs) appears normal.

Magnification 23,000 x.

Fig. 4.13B A cell (t), which resembles a thrombocyte, escaping through a gap (g) in the endothelium (e) of a small thin-walled vessel. Red blood cells (r) remain within the vessel lumen.

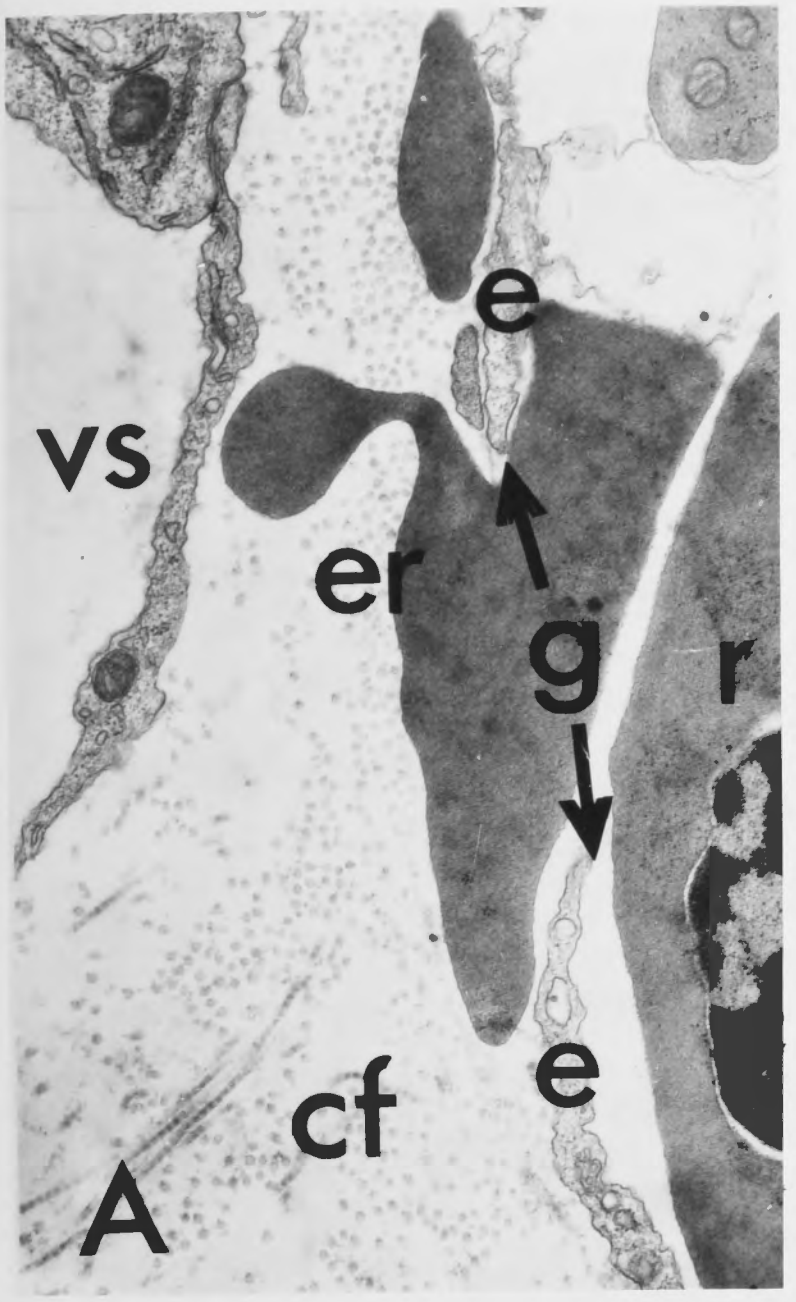
Magnification 23,000 x.

Fig. 4.13C A microthrombus (mth) among the extravasated red blood cells (r) in the mesenchymal layer is shown in this micrograph.

Magnification 23,000 x.

Fig. 4.13D A small capillary vessel (vs) which appears undamaged, within the chorionic epithelial layer (ce). An endothelial cell (e) protrudes into the vascular lumen which contains a red blood cell (r). Some epithelial cells in the chorionic epithelium have degenerated (de) due to injury caused by 'dropping' the CAM. The underlying mesenchymal layer contains long processes from mesenchymal cells (m).

Magnification 9,300 x.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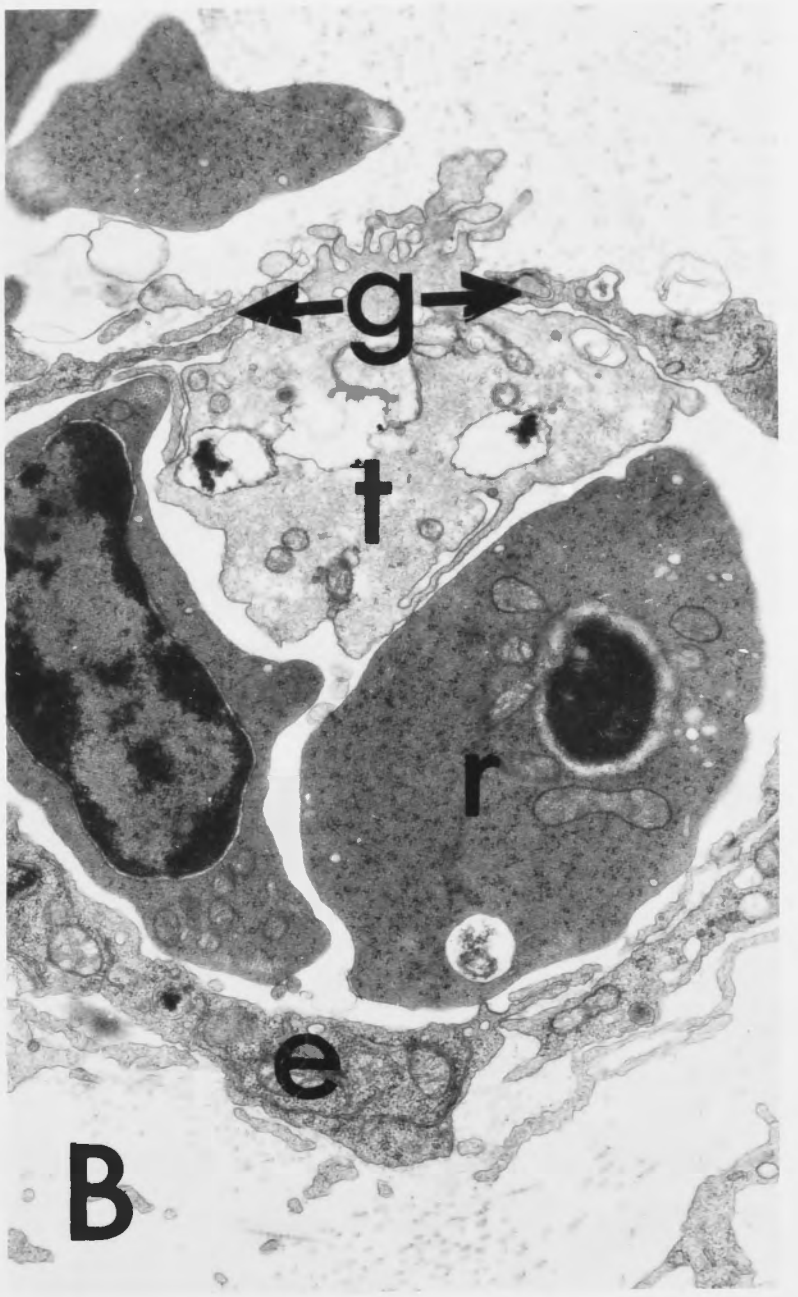
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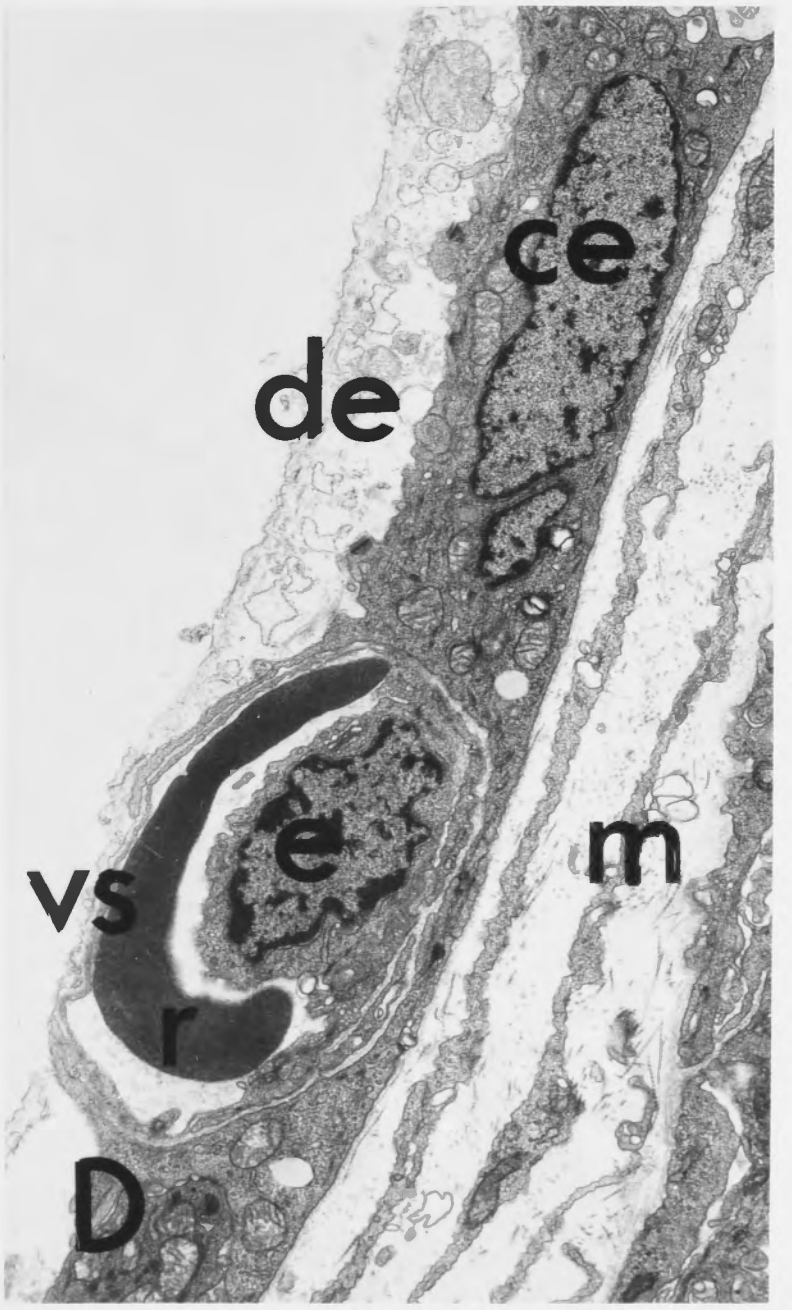
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even in areas where large numbers of extravasated red blood cells were present in the underlying mesenchyme. A typical small capillary from the chorionic epithelium is shown in fig. 4.13D. The endothelial cell bulges into the vessel lumen but the endothelial cytoplasm is normal in appearance and the endothelial layer shows no degenerative changes. Some superficial epithelial cells of the chorionic epithelium have been damaged, but this is a local effect due to injury caused when the CAM is 'dropped' before inoculation.

Discussion

The experiments described in this chapter demonstrate that a GVHR can be induced in young (4-6 day) recipients. In contrast to the proliferative lesions which characterise the GVHR in older recipients (Simonsen, 1957; Biggs and Payne, 1961a, b) widespread haemorrhages occur. These haemorrhagic lesions cannot be induced by syngeneic adult donor cells or by embryonic allogeneic cells and are therefore similar in their requirements to proliferative changes such as splenomegaly which occur during a GVHR in older hosts (Simonsen, 1957, 1962). Thus, pathological changes associated with a GVHR appear to be strongly influenced by recipient age and the absence of proliferative lesions such as spleen enlargement and pock formation in very young hosts cannot be taken as lack of a GVHR.

The reason for the development of haemorrhagic lesions during a GVHR in these young embryos is not clear. Haemorrhages have been described in chick embryos subjected to various forms of injury including hypoxia (Grabowski, 1966) and X-irradiation (Stearner and Sanderson, 1969). They are also seen after treatment with influenza virus (Hooke and Wagner, 1958; Hooke *et al.*, 1958), Rous sarcoma virus (Sweeny and Bather, 1968) bacterial endotoxins (Smith and Thomas, 1956; Hooke *et al.*, 1961) and allantoic fluid (Simonsen, 1970b). However, in most of these cases, haemorrhages appeared very rapidly, while in embryos inoculated with allogeneic blood, they may take several days to develop. The widespread nature and relatively late appearance of these haemorrhagic lesions in the GVHR suggest that they may be a secondary development rather than the

result of a direct effect of donor cells on the host endothelium. They may be caused by the appearance of injurous substances within the circulation.

This suggestion is strengthened by the similarity to vascular damage which occurs after other forms of injury such as X-irradiation (Stearner and Sanderson, 1969) or in the delayed response which follows thermal injury (Cotran, 1967).

In the blood vessels in the area pellucida of the 3 day chick embryo subjected to X-irradiation (1,000-2,000R) injury ranges from minor changes involving irregularities of the cell surface and the projection of cells into the vessel lumen to severe damage of the endothelial cells which often develop an abnormally dense cytoplasm. Eventually cell lysis leads to a complete disruption of the vessels (Stearner and Sanderson, 1969). These changes are very similar to those seen in vessels of the digit from very young embryos undergoing a GVHR.

Whatever the cause of vessel damage, haemostatic mechanisms normally operate to stop blood loss (Hjort and Hasselback, 1961). In adult mammals, 3 processes are normally involved : contraction of the vessel wall, formation of a platelet plug at the site of injury and formation of a fibrin clot (Hughes-Jones, 1970).

The blood of early chicken embryos, however, does not clot (Pickering and Gladstone, 1925; Kane and Sizer, 1953) and the embryo appears to rely on thrombus formation to control haemostasis (Lemež, 1964). This lack of clotting ability is due to the absence of fibrinogen and thrombin (Pickering and Gladstone, 1925). None of the major clotting components are present in the blood of 9 day embryos, but fibrinogen and prothrombin are present by 11 days (Kane and Sizer, 1953). A deficiency in the thrombin-prothrombin conversion mechanism in the blood of 11 day embryos, however, still prevents thrombin from being formed and clotting does not occur until the embryo is 12 or 13 days old (Kane and Sizer, 1953).

Avian thrombocytes develop at a very early stage of embryonic life and are first recognizable at 48 hours (Stalsberg and Prudz, 1963a). They appear to have similar

haemostatic properties to mammalian platelets (Stalsberg and Prudz, 1963b) and vascular damage in chick embryos is normally quickly followed by the formation of a haemostatic plug, composed almost exclusively of thrombocytes (Edmonds, 1968, 1970). The time required for the cessation of haemorrhage by the formation of a thrombocyte plug varies with the size of the vessel but does not vary with the age of the embryo. Embryos examined between 3 and 18 days incubation did not vary significantly in bleeding time (Edmonds, 1968).

Thrombus formation has been described in many forms of vessel injury (Stearner and Sanderson, 1969; Ashford and Freiman, 1967; Cotran, 1967) but it was not seen in vessels of embryos with haemorrhagic lesions caused by a GVHR, although thrombocytes were occasionally seen escaping into the connective tissue or among extravasated red blood cells. No thrombi were found within the vascular lumen.

This suggests that these embryos may be thrombocytopenic. Thrombocytopenia is frequently associated with haemorrhagic disorders (Rosenthal, 1938). Gaydos and coworkers (1966), for example, have shown that the severity and frequency of haemorrhage in leukemic patients increases with declining platelet levels. However, thrombocytopenia alone rarely produced haemorrhage unless there is a simultaneous interference with other haemostatic mechanisms (Rosenthal, 1938). This has been demonstrated by the experiments of Calaresu and Jaques (1960) who showed that the mortality in thrombocytopenic rats from spontaneous haemorrhage was very much greater when they were also treated with dicumerol to produce prothrombopenia or when they were given NaCl to produce stress.

If thrombocytopenia develops in very young chick embryos before the blood clotting system has developed, they may become very susceptible to haemorrhage. The presence of deficiencies in several components of the haemostatic system early in embryonic life, may explain why haemorrhage occurs during a GVHR in very young embryos but is not seen in older recipients.

The lack of structural differentiation of the blood vessels at an early stage may also contribute to the develop-

ment of haemorrhage during the GVHR in very young embryos. Exposed collagen (Kjaerheim and Hovig, 1962; Spaet and Zucker, 1964; Jamieson et al., 1971) and basement membrane material (Hughes and Mahiew, 1970) have been shown to initiate platelet aggregation. Most of the vessels in the digits of the developing embryo were found to lack a basement membrane and only a few collagen fibres were seen in the surrounding connective tissue. In the CAM, where many blood vessels are well differentiated, haemorrhage was found to occur from capillaries and other thin walled vessels, but not from thick walled vessels.

These features : the absence of a clotting mechanism, possible thrombocytopenia and poorly differentiated blood vessels with little or no basement membrane material or collagen around them may, therefore, all contribute to the development of haemorrhage in the young chick embryo once a GVHR has been initiated.

CHAPTER 5

CHANGES IN LYSOSOMAL ENZYME ACTIVITY DURING A GRAFT-VERSUS-HOST REACTION

CHAPTER 5. CHANGES IN LYSOSOMAL ENZYME ACTIVITY DURING A GRAFT-VERSUS-HOST REACTION

Introduction

The changes in lysosomal enzyme activity which occur in the spleen of normal chick embryos and in the spleen of chick embryos undergoing a graft-versus-host reaction (GVHR) have been examined by Kimmel (1967a, b, c). In the normal spleen there is a marked rise in lysosomal enzyme content between 12 and 16 days incubation (Kimmel, 1967a). DeLanney and co-workers (1962) had previously shown that during this time the spleen develops as a reticuloendothelial organ and granulopoiesis commences. This led Kimmel (1967a) to suggest that the increase of lysosomal enzyme activity in the spleen at this stage was due to an increased number of granulocytes and macrophages, cells which are known to have a high content of lysosomal enzymes (Archer and Hirsch, 1963; Cohn and Hirsch, 1960; Cohn and Wiener, 1963; Osculati, 1970).

Graft-versus-host reactions in the chick embryo cause a large rise in granulopoietic activity in the spleen (DeLanney et al., 1962). A significant increase in the content of 3 lysosomal enzymes : β-glucuronidase, acid phosphatase and acid deoxyribonuclease was also noted in the enlarged spleens of chick embryos which had been inoculated at 9 or 12 days of age with adult leukocytes or spleen cells (Kimmel, 1967b, c). This coincided with an augmented number of cells staining positively for acid phosphatase (Kimmel, 1967c). It was therefore suggested that the host's response during a GVHR might involve the mobilization of phagocytic cells, leading to an increase in the activity of lysosomal enzymes in the spleen.

The experiments described in previous chapters have shown that there is a marked difference in the pathogenesis of GVHRs depending on the age of the host : haemorrhagic lesions develop in embryos inoculated with adult allogeneic blood before 6 days of incubation, while embryos inoculated at a later stage developed proliferative lesions. It therefore seemed of interest to examine whether corresponding differences existed in lysosomal

Table 5.1

Units of N-acetyl glucosaminidase activity in the spleen of randomly bred embryos, 6 days after the inoculation of 0.1 ml of diluted, adult AA blood onto the CAM. Control embryos were inoculated with physiological saline. Mean values and standard errors are given for the enzyme activity in each group. The units of activity are expressed at µg para-nitrophenol released per hour per mg (wet weight) of spleen tissue.

Age at inoculation	5 days	6 days	8 days	10 days	14 days
Experimental group	1.7 ± 0.3	1.9 ± 0.2	1.8 ± 0.4	2.1 ± 0.3	2.4 ± 0.6
Control group	1.2 ± 0.2	1.1 ± 0.1	1.0 ± 0.2	1.6 ± 0.3	2.9 ± 0.5
Average increase above control level	0.5	0.8	0.8	0.5	none
t-Test probability	0.17	0.02*	0.05*	0.32	0.54

* significant at the 5% level

enzyme levels during the development of this disease.

Lysosomal enzyme activity in a tissue is generally related to the number of lysosome-rich cells, while increased enzyme levels in the tissue fluids are thought to result from cell injury which causes rupture of the lysosomal membranes followed by leakage of hydrolytic enzymes into the surrounding tissue space (de Duve, 1964).

In the following experiments, spleens from 5 to 14 day old recipients were assayed for an increase in tissue-bound lysosomal enzyme activity associated with a GVHR, while the plasma from very young embryos with haemorrhagic lesions was assayed for increased lysosomal enzyme activity associated with cell damage.

Experimental results

The effect of recipient age on lysosomal enzyme changes in the spleen of chick embryos undergoing a graft-versus-host reaction

Randomly bred chick embryos of 5 to 14 days incubation were inoculated on the CAM with diluted adult AA blood (0.1 ml of a 1:1 dilution of adult AA blood in Alsever's solution) and their spleens were assayed 6 days later for N-acetyl glucosaminidase (chapter 2). Eight to 12 embryos were used in each group.

Table 5.1 shows that there was a significant increase, above control level, in N-acetyl glucosaminidase activity in the spleens of embryos inoculated at 6 and 8 days but not in those inoculated either at 5 days or at 10 or 14 days.

In embryos inoculated up to 8 days of age, there appeared to be a correlation between spleen enlargement and increased N-acetyl glucosaminidase activity. In chapter 3 (table 3.1) it was shown that inoculation of adult allogeneic blood into 5 day old embryos did not cause splenomegaly. In comparable animals of this series there was no significant increase in spleen N-acetyl glucosaminidase activity. In those inoculated on the 6th or 8th day of incubation, the spleen had enlarged significantly and there was a concomitant rise in N-acetyl glucosaminidase activity.

In contrast, in embryos inoculated on day 10 or day 14 which had the largest percentage increase in spleen weight (table 3.1), N-acetyl glucosaminidase activity in the spleen was not correspondingly elevated (table 5.1). In order to determine whether N-acetyl glucosaminidase activity was in fact not correlated with spleen weight in this age group or whether the timing of the enzyme assay missed the peak of enzyme activity, further assays were made 2 to 6 days after the inoculation of allogeneic blood.

Changes in lysosomal enzyme activity in the spleen of 10 day recipients

Randomly bred embryos were inoculated on the CAM at 10 days with diluted adult AA blood and their spleens were weighed and assayed for N-acetyl glucosaminidase activity 2, 3 and 4 days later. Five to 7 spleens were used in each group. The results of these assays are shown in tables 5.2 and 5.3.

Table 5.2

Units of N-acetyl glucosaminidase activity in the spleen of 12 to 16 day randomly bred embryos inoculated on the CAM at day 10 with diluted, adult AA blood. Mean values and standard errors are given for the enzyme activity in each group. The units of activity are expressed as µg para-nitrophenol released per hour per mg (wet weight) of spleen.

Days after inoculation	2 days	3 days	4 days	6 days†
Experimental group	3.2 ± 0.4	2.6 ± 0.4	2.6 ± 0.1	2.1 ± 0.3
Control group	1.1 ± 0.3	1.2 ± 0.4	1.2 ± 0.3	1.6 ± 0.3
Average increase above control level	2.1	1.4	1.4	0.5
t-Test probability	0.0016*	0.0309*	0.0026*	0.3200

† as in table 5.1
* significant at the 5% level

As can be seen (table 5.2) a significant increase in N-acetyl glucosaminidase activity had occurred in the spleen by the 2nd, 3rd and 4th day but was no longer present 6 days after inoculation.

Table 5.3

Wet weights of the spleens taken from 12 to 16 day old randomly bred embryos after inoculation on the CAM at 10 days with diluted AA adult blood. Mean values with the standard error for each group are given in mg.

Days after inoculation	2 days	3 days	4 days	6 days
Experimental group	7.6 ± 0.4	9.4 ± 0.4	15.0 ± 1.1	36.5 ± 4.3
Control group	7.4 ± 0.5	9.5 ± 0.5	10.3 ± 1.0	12.9 ± 0.7
Average increase above control level	0.2	none	4.7	23.6
t-Test probability	0.8141	0.8632	0.0090*	0.0028*

* significant at the 5% level

The average spleen weight of these embryos, however, does not become significantly higher than that of the Controls until 4 days and is maximal after 6 days. This confirmed that the increase in lysosomal enzyme activity did not coincide with the increase in spleen weight in this age group.

Further tests were made to determine whether the apparent correlation of increased N-acetyl glucosaminidase activity with spleen enlargement in younger age groups tested at 6 days (table 5.1) was also present in earlier stages of the disease.

Changes in lysosomal enzyme activity in the spleen of 6 day recipients

The spleens of embryos inoculated at 6 days of age were weighed and assayed for N-acetyl glucosaminidase activity 4 days later, 5 to 6 embryos were examined in each group. The results of these assays are shown in tables

5.4 and 5.5.

Table 5.4

Units of N-acetyl glucosaminidase activity in the spleen of 10 and 12 day randomly bred embryos inoculated on the CAM at 6 days with diluted adult AA blood. Mean values and standard errors are given for the enzyme activity in each group.

Activity is expressed as µg para-nitrophenol released per hour per mg (wet weight) of spleen tissue.

Days after inoculation	4 days	6 days†
Experimental group	0.20 ± 0.04	1.9 ± 0.2
Control group	0.13 ± 0.03	1.1 ± 0.1
Average increase above control level	0.07	0.8
t-Test probability	0.6374	0.0200*

† as in table 5.1
* significant at the 5% level

Table 5.5

Wet weights (mg) of the spleens from 10 and 12 day randomly bred embryos after inoculation on the CAM at 6 days with diluted adult AA blood. Mean values with the standard error for the experimental and control groups are given.

Days after inoculation	4 days	6 days
Experimental group	6.3 ± 0.8	10.2 ± 0.9
Control group	4.3 ± 0.9	7.4 ± 0.4
Average increase above control level	2.0	2.8
t-Test probability	0.1419	0.0075*

* significant at the 5% level

Table 5.6

Units of enzyme activity in the plasma of 12 day old CC embryos inoculated on the CAM with 0.1 ml of diluted adult AA blood. Mean values and standard errors are given for acid phosphatase, β-glucuronidase, acid ribonuclease and N-acetyl glucosaminidase.

Enzyme	Units of activity		Difference	t-Test probability
	Experimental group	Control group		
Acid phosphatase[1]	7.7 ± 0.5	3.4 ± 0.4	4.3	0.0003*
β-glucuronidase[2]	0.43 ± 0.05	0.20 ± 0.04	0.23	0.0005*
Acid ribonuclease[3]	1.56 ± 0.20	0.78 ± 0.13	0.78	0.0175*
N-acetyl glucosaminidase[1]	11.5 ± 2.5	5.9 ± 0.5	7.6	0.0300*

[1]Activity expressed as µg para-nitrophenol released per hour per 0.1 ml plasma.

[2]Activity expressed as µg phenolphthalein released per hour per 0.1 ml plasma.

[3]Activity expressed in terms of the change in optical density at 260 nm per hour per 0.1 ml plasma.

*Significant at the 5% level.

In contrast to embryos inoculated at 10 days of age, the increase in N-acetyl glucosaminidase in the spleen of 6 day recipients does not become significantly elevated above control level until 6 days after the inoculation of adult blood. This coincides with a significant increase of spleen weight in these embryos (table 5.5).

Lysosomal enzyme activity in the yolk sac

In chapters 6 and 8, the pathological changes which occur in the yolk sac during a GVHR will be described. It was found that there is a severe cell depletion in the blood islands, but proliferative lesions do not develop.

Yolk sac tissue was therefore examined in order to determine whether an increase in N-acetyl glucosaminidase occurs in the absence of proliferative lesions.

Randomly bred embryos of 5 to 14 days were inoculated on the CAM with diluted adult AA blood and the activity of N-acetyl glucosaminidase in the yolk sac was determined 6 days later. The dry weight of each yolk sac was also recorded. Ten to 12 embryos were examined in each group.

Fig. 5.1A shows that the N-acetyl glucosaminidase activity in these recipients did not differ from that of control embryos and there was similarly no increase in the dry weight of the yolk sac (fig. 5.1B).

Lysosomal enzyme activity in the plasma of inbred CC embryos after the inoculation of allogeneic adult blood at 6 days incubation

Six day old CC embryos were inoculated on the CAM with diluted adult AA blood. Control embryos were inoculated in a similar manner with 0.1 ml of physiological saline. Six days after inoculation 0.2-0.4 ml of blood was withdrawn from an allantoic vein and the plasma was examined for acid phosphatase, β-glucuronidase, N-acetyl glucosaminidase and acid ribonuclease activity as described in chapter 2. Plasma samples from 6 to 10 embryos were examined for each enzyme assay. The results of these assays are presented in table 5.6.

The levels of all 4 lysosomal enzymes examined were increased in the plasma of the experimental group. These increases were significant at the 5% level in a Student t-test. This experiment was repeated with syngeneic blood

Fig. 5.1A A comparison of the average N-acetyl glucosaminidase activity of the yolk sac from normal 12 to 20 day old chick embryos (open circles) and in the yolk sac of experimental animals of the same age inoculated 6 days previously with adult allogeneic blood (closed circles). N-Acetyl glucosaminidase activity is expressed as µg paranitrophenol released per hour per mg yolk sac (dry weight). The vertical lines represent the standard error. The graph shows that there is no significant difference between control and experimental embryos in any age group.

Fig. 5.1B A comparison of the average dry weight of the yolk sac in normal embryos (open circles) and in experimental animals of the same age inoculated 6 days previously with adult allogeneic blood (closed circles). The vertical lines represent the standard error of the mean. There was no significant difference in yolk sac dry weight between experimental and control animals of any age group.

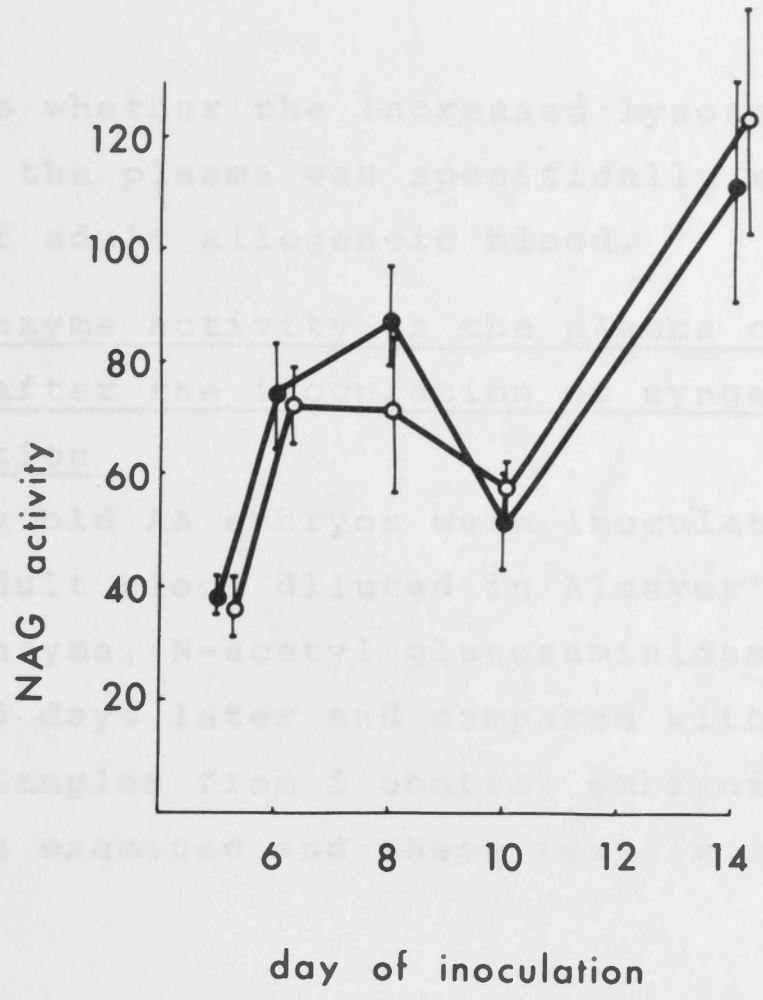


fig. 5.1 A

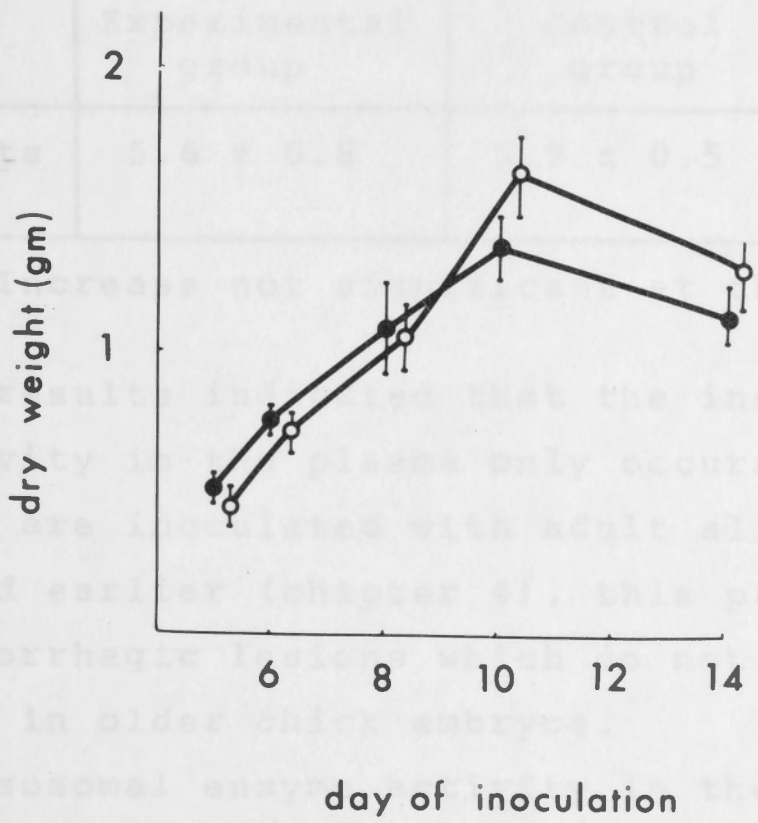


fig. 5.1 B

to determine whether the increased lysosomal enzyme activity in the plasma was specifically related to the inoculation of adult allogeneic blood.

Lysosomal enzyme activity in the plasma of inbred AA recipients after the inoculation of syngeneic blood at 6 days incubation

Six day old AA embryos were inoculated on the CAM with syngeneic adult blood diluted in Alsever's solution. The lysosomal enzyme, N-acetyl glucosaminidase, was assayed in the plasma 6 days later and compared with plasma levels of controls. Samples from 5 control embryos and 8 experimental animals were examined and these results are presented in table 5.7.

Table 5.7

N-Acetyl glucosaminidase activity in the plasma of 12 day old AA chick embryos inoculated on the CAM at 6 days with 0.1 ml of diluted adult AA blood. Mean values and standard errors are given for the enzyme activity in each group. N-Acetyl glucosaminidase activity is expressed in µg para-nitrophenol released per hour per 0.1 ml of plasma.

	Enzyme activity		t-Test probability
	Experimental group	Control group	
AA recipients	5.6 ± 0.8	5.9 ± 0.5	0.76**

** Increase not significant at the 5% level

These results indicated that the increase in lysosomal enzyme activity in the plasma only occurs when chick embryos of this age are inoculated with adult allogeneic blood. As described earlier (chapter 4), this procedure induces severe haemorrhagic lesions which do not occur if a GVHR is produced in older chick embryos.

The lysosomal enzyme activity in the plasma of 10 day CC chick embryos was examined in order to determine whether a similar increase occurred in older recipients.

Table 5.8

Units of enzyme activity in the plasma of 16 day old CC embryos inoculated on the CAM at 10 days with 0.1 ml of diluted adult AA blood. Mean values and standard errors are given for 4 lysosomal enzymes.

Enzyme	Units of activity		Difference	t-Test probability
	Experimental group	Control group		
Acid phosphatase[1]	5.1 ± 0.9	3.1 ± 0.4	2.0	0.1**
β-glucuronidase[2]	0.55 ± 0.14	0.46 ± 0.11	0.09	0.6**
Acid ribonuclease[3]	0.69 ± 0.12	0.27 ± 0.05	0.42	0.07**
N-acetyl glucos-aminidase[1]	14.2 ± 1.3	11.7 ± 0.9	2.5	0.15**

[1]Activity expressed as µg para-nitrophenol released per hour per 0.1 ml plasma.

[2]Activity expressed as µg phenolphthalein released per hour per 0.1 ml plasma.

[3]Activity expressed in terms of the change in optical density of 260 nm per hour per 0.1 ml plasma.

**Not significant at the 5% level.

Lysosomal enzyme activity in the plasma of inbred CC embryos after the inoculation of allogeneic blood at 10 days

Ten day old CC embryos were inoculated on the CAM with diluted adult AA blood and examined 6 days later. All embryos had developed pocks on the CAM and showed a significant increase above control level in spleen weight. No haemorrhages were seen.

Plasma from these embryos were assayed for N-acetyl glucosaminidase, acid phosphatase, acid ribonuclease and β-glucuronidase activity. The values obtained were compared with those of control embryos which had been inoculated at the same age with physiological saline. Samples of plasma from 6 to 8 embryos were used for each enzyme assay in each group.

Table 5.8 shows that there was no significant difference between the enzyme activity in the plasma of the control and the experimental group for any of the 4 lysosomal enzymes assayed.

It can be concluded that the increase of lysosomal enzyme activity in the plasma which occurs when 6 day old embryos are inoculated with allogeneic blood is related to the development of haemorrhagic lesions.

Discussion

The increase in lysosomal enzyme activity in the spleen during a GVHR has been attributed to an increase in the number of granulocytes and macrophages which are known to be rich in lysosomal enzymes (Kimmel, 1967c). A similar rise in lysosomal enzyme activity associated with augmented numbers of macrophages in lymphoid tissue has also been reported in wasting disease following thymectomy in mice (Anton and Brandes, 1969).

Spleen enlargement during the GVHR is thought to be due to the combined effect of the proliferation of primitive reticulum cells and augmented granulopoiesis (Biggs and Payne, 1961a, b). If this is so, then the present experiments indicate that the age of the host greatly affects the relative intensity of reticulum cell or granulocyte response. In young recipients (6-8 days) tissue-bound lysosomal enzyme activity rises as the spleen enlarges. In recipients aged 10 to 14 days, lysosomal

enzyme activity has reached a peak and declined before splenomegaly is maximal. Thus in the first group, both systems appear to respond at the same time, while in the second, granulopoiesis appears to predominate in early stages of the disease and is then overtaken by reticulum cell proliferation.

The effect of recipient age on the pathogenesis of a GVHR was reflected in lysosomal enzyme levels, even in very young embryos. As discussed in chapter 4, widespread haemorrhages, rather than proliferative lesions, develop in response to the inoculation of adult allogeneic blood. Tissue-bound lysosomal enzyme activity in the spleen of very young embryos was not elevated. However, there was a significant increase in the plasma levels of lysosomal enzymes which could not be detected in animals with proliferative lesions.

This agrees well with the degenerative changes described in chapter 4 and the concept that increased levels of lysosomal enzymes in the tissue fluids reflects cell injury (de Duve, 1964).

Lysosomes, which are concerned with the digestion of heterologous and autologous material (de Duve and Wattiaux, 1966; de Duve, 1969; Weissman and Dukor, 1970) are very susceptible to tissue injury and rupture of the lysosomal membrane may lead to leakage of hydrolytic enzymes from damaged cells (de Duve, 1964). In the present experiments it is not known whether vascular damage is caused by the presence of circulating hydrolytic enzymes or whether their appearance in the blood is secondary to the vascular injury. So far it has not been established whether lysosomal rupture can itself initiate cell damage *in vivo* (Slater, 1969).

For example, hydrolytic enzyme activity does not appear to be important in the early stages of CCl_4 or dimethyl nitrosamine induced necrosis, or in ischaemia (Slater, 1969).

Experiments in this chapter have demonstrated that graft-versus-host reactions of the proliferative type are associated with increased levels of tissue-bound lysosomal enzymes while the degenerative lesions which are character-

istic of this disease in very young embryos relate to increased lysosomal enzyme activity in the plasma.

CHAPTER 6

PATHOLOGICAL CHANGES IN THE YOLK SAC DURING THE GRAFT-VERSUS-HOST REACTION IN VERY YOUNG EMBRYOS

CHAPTER 6. PATHOLOGICAL CHANGES IN THE YOLK SAC DURING THE GRAFT-VERSUS-HOST REACTION IN VERY YOUNG EMBRYOS

Introduction

Inoculation of 6 day old inbred CC embryos with adult allogeneic blood causes petechial haemorrhages to appear on the body surface as described in chapter 4. Since this pathological state was frequently accompanied by changes in the yolk sac, a closer examination of this tissue was carried out.

Normal development of the yolk sac

The yolk sac of the chick embryo develops from an ectodermal and endodermal sheet known as the area vitellina which gradually extends over the surface of the yolk. During this process it is invaded centrifugally by mesoderm to form the area vasculosa (Romanoff, 1960).

The first development of haemopoietic tissue in the yolk sac occurs at the 11 somite stage when local thickenings, produced by the differentiation of angioblasts from mesenchymal cells, appear in a horse-shoe shaped area around the caudal end of the embryo (Sabin, 1917). These angioblasts give rise both to the earliest blood cells and to vascular endothelium, a process that has been studied by a variety of methods (Sabin, 1917, 1920, 1921; Danchakoff, 1908, 1916c; Sugiyama, 1926; Murray, 1932) including phase and electron microscopy (Houser *et al.*, 1961; Edmonds, 1966; Gonzalez-Crussi, 1971).

Developing angioblasts, which are more granular and refractile than the surrounding mesenchymal cells (Sabin, 1917) form clusters of cells, attached by desmosomes (Edmonds, 1964) which eventually form solid cords. These, in turn, anastomose to form a network (Houser *et al.*, 1961). A lumen is then formed within each cord by widening and coalescence of intercellular spaces (Edmonds, 1966; Gonzalez-Crussi, 1971) rather than by degeneration of centrally located angioblasts as previously suggested (Sabin, 1920; Houser *et al.*, 1961). Outer cells of the cord give rise to endothelium, while cells trapped within the lumen, constitute the blood islands (Sabin, 1920). These early blood islands contain large immature cells with

a cytoplasm that contains many free ribosomes and very little endoplasmic reticulum (Edmonds, 1966). They appear to be primitive multipotential stem cells which give rise to cells of the primitive erythrocytic series and later also to thrombocytes (Sugiyama, 1926; Edmonds, 1966). As the differentiation of blood vessels progresses these primitive stem cells also appear in perivascular areas (Edmonds, 1966) where they give rise to granulocytes (Danchakoff, 1908, 1916c).

During the development of haemopoietic tissue, a circulatory system is established within the area vasculosa by differentiation of angioblastic cords (Sabin, 1920) and further growth of vascular sprouts (Sabin, 1922). Blood begins to flow through this indifferent capillary network at the 16 somite stage (Hughes, 1935). Taking a path of least resistance (Hughes, 1937), it percolates away from the body to the periphery of the membrane where it collects in vascular spaces and returns anteriorly to the heart. These peripheral vascular spaces coalesce at a later stage to form the marginal sinus, which now drains into 2 anterior vitelline veins returning to the heart. Definite arterial vessels first develop at the end of the second day when the vitelline arteries differentiate on each side of the embryo, in the area where blood from the dorsal aorta flows rapidly into the capillary network in the yolk sac (Hughes, 1935).

At the end of the third day, the primary circulation of the yolk sac has become established (Romanoff, 1960). The vitelline arteries have become extensively branched and posterior and lateral veins also have developed from the capillary network in the caudal region providing a more direct venous return. The marginal sinus now diminishes in importance and gradually regresses (Popoff, 1894) while one of the anterior veins, which it supplies, atrophies.

The definitive circulation (Romanoff, 1960) which develops in close association with the yolk sac folds (Remotti, 1927) also begins to differentiate during the third day. Longitudinal folds of the endoderm, closely associated with branches of the vitelline arteries extend

into the substance of the yolk. It is not clear whether these folds develop autonomously to provide an increased area for yolk absorption or whether they form secondarily around the arterial vessels which tend to sink below the surface. Bifurcations of folds, however, follow the vascular ramifications (Remotti, 1927).

With further development, these folds become thin leaves suspended from the upper surface of the yolk sac. An arterial vessel courses through the core of the thickened peripheral margin of the fold and becomes surrounded by a dense plexus of venous capillaries which communicate with the superficial network in the upper surface of the yolk sac by a series of long straight vessels with ladder-like anastomoses (Popoff, 1894). Intense haemopoietic activity has been described in this peri-arterial vascular network (Danchakoff, 1908, 1916c; Willier, 1968). Romanoff (1952) has also pointed out its importance in the transport of nutrients absorbed from the yolk.

There are some ambiguities in the description of the relationship of blood flow through the marginal artery of the fold and that through the surrounding venous plexus. The arteries are said to follow the entire length of the fold, often a distance of up to 4-5 cm without giving off any branches (Popoff, 1894), while no mention is made of the vessels which supply the peri-arterial venous plexus. For this reason, normal yolk sacs as well as those from embryos with GVHR's were studied.

Experimental results

Normal development of the yolk sac and its folds

The development of yolk sac folds was examined in normal randomly bred chick embryos. The yolk sac was removed, washed in saline and photographed unstained, either from the yolk aspect to show the arteries and folds, or from the upper surface to show the superficial vascular system.

The yolk sac of a 4 day chick embryo photographed from the yolk aspect is shown in fig. 6.1A. The vitelline arteries are already well differentiated at this stage and project from the plane of the membrane. Early stages of

Fig. 6.1A-C The development of the yolk sac in the chick embryo from 4 to 7 days.

Fig. 6.1A Yolk sac from a 4 day old chick embryo photographed from the yolk aspect. The vitelline arteries (a) and some early folds, especially prominent around some arterial branches on the right, appear raised. The embryo is marked (e).

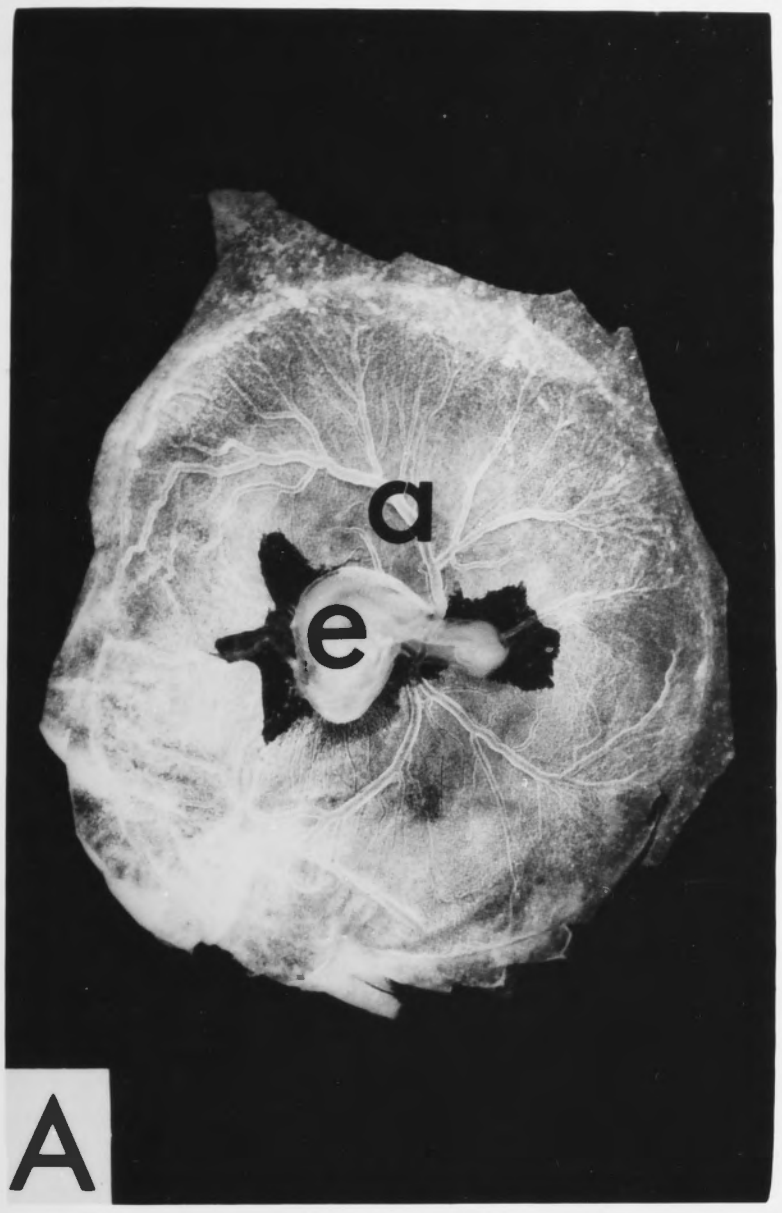
Magnification 2.3 x.

Fig. 6.1B Superficial aspect of a yolk sac at 5 days showing the major venous vessels including the anterior (av) and posterior (pv) vitelline veins, intermediate (i) and collateral veins (c) and the marginal sinus (s). The embryo is marked (e). See text.

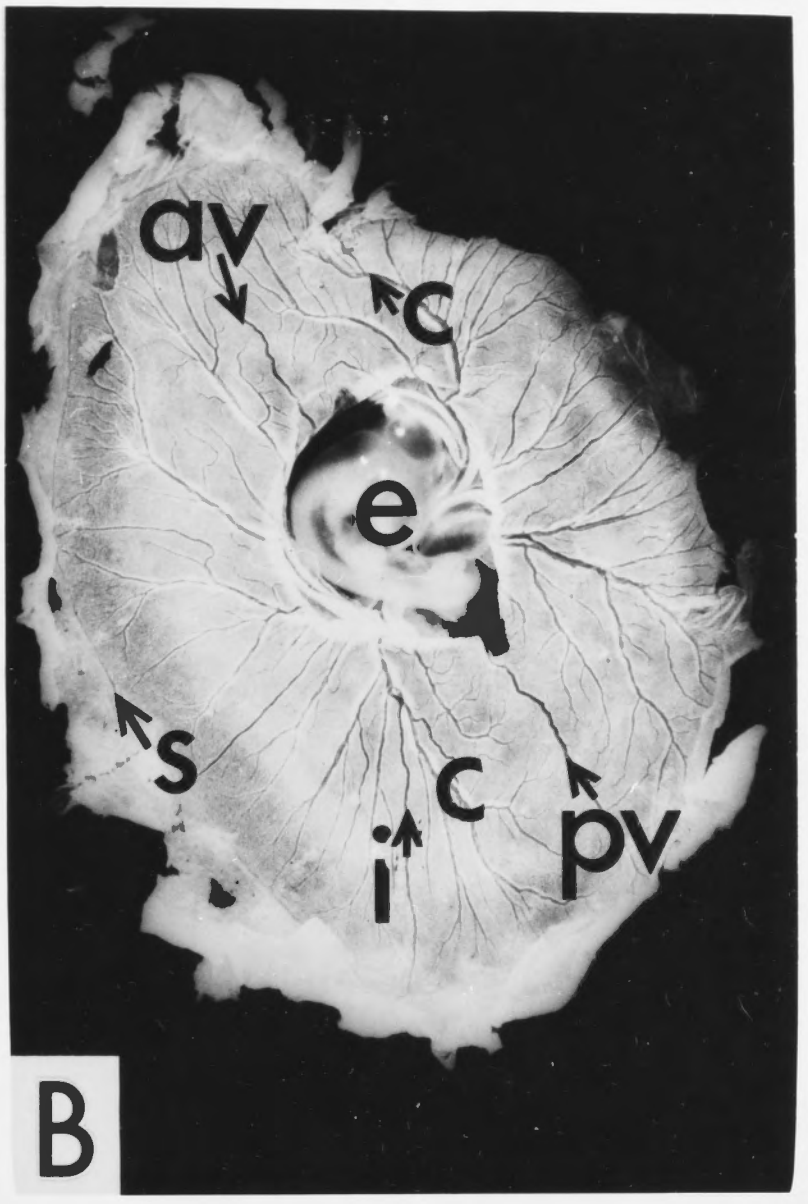
Magnification 2.3 x.

Fig. 6.1C Yolk sac of a 7 day chick embryo again photographed from the superficial aspect to illustrate the extensive branching of the folds (f) which follow the arterial vessels. Collateral veins (c) run above the arteries while the intermediate veins (i) drain the area between the folds. The embryo is marked (e).

Magnification 1.5 x.



A

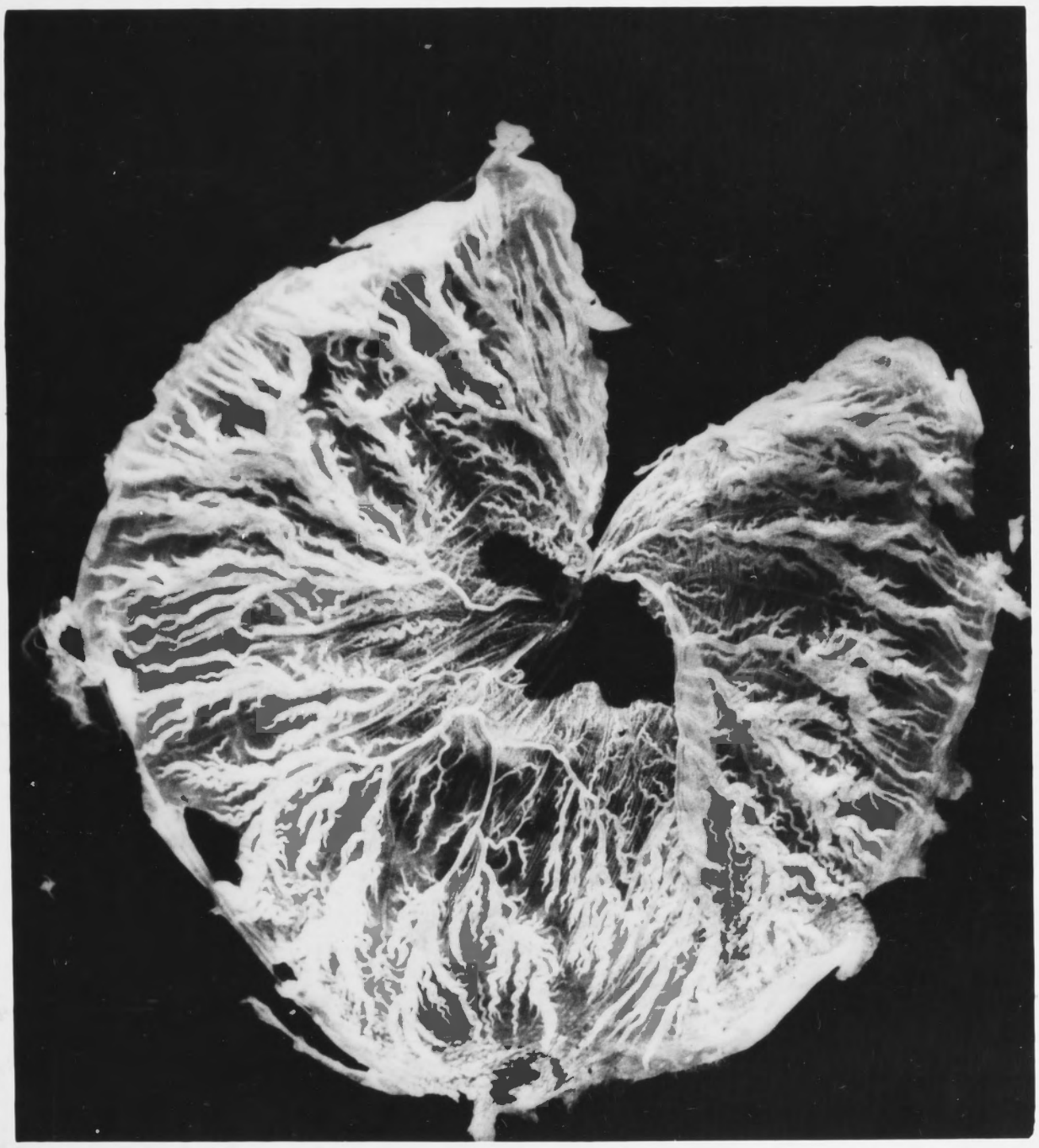


B

C

Fig. 6.2 The yolk sac of a 10 day old chick embryo photographed from the yolk aspect to illustrate the development of the yolk sac folds at this stage. The folds now branch profusely to follow the ramifications of the vitelline arteries. They now extend further into the yolk, becoming highest in the equatorial region of the yolk sac where their margins become sinuous.

Magnification 1.5 x.



folds are present around some of the arterial branches.

The superficial venous network of the yolk sac in the 5 day chick embryo is illustrated in fig. 6.1B. The anterior and posterior vitelline veins are well developed and return blood from the peripheral sinus. Collateral veins lie above the arterial vessels and follow the same course. Intermediate veins run between the developing folds and drain the area between two adjacent arteries. Both the intermediate and the collateral veins drain into lateral vitelline veins at each side of the embryo. As illustrated in fig. 6.1C, the yolk sac folds have increased in height and complexity by the seventh day as they follow the many ramifications of the arterial vessels. This development continues so that by 10 days (fig. 6.2) a profuse system of folds has differentiated. The yolk sac folds are now extensively branched. They are highest in the equatorial region of the membrane where their margins become sinuous.

The development of the yolk sac folds was examined in more detail in whole mount preparations of yolk sac from randomly bred chick embryos of 5 to 12 days. Figs. 6.3A-E illustrate the gradual progression from slightly raised ridges around the arterial vessels (fig. 6.3A) to deep wavy folds in the later stages. At 12 days (fig. 6.3E) the folds project far into the yolk and the free margin of the fold, which is moulded by the underlying vascular network, has become thicker and more irregular in outline.

The circulatory system within the yolk sac fold

The circulatory system within the yolk sac folds was examined in the yolk sac of 15 day old normal chick embryos injected with a gelatine injection mass containing colloidal carbon.

At this stage, the yolk sac is at its height of development. A dense capillary and venous network permeates the outer layer (fig. 6.4C) of this membrane and its folds (fig. 6.4A) which project into the yolk. An arterial vessel runs along the free margin of each fold. Surrounding this artery is an extremely dense plexus of fine vessels (fig. 6.4A) which communicates with the superficial network by a series of somewhat longer and straighter vessels. The

Fig. 6.3A-F The development of the yolk sac fold from 5 to 12 days incubation as seen in a series of whole mounts, stained with osmium and photographed from the yolk aspect. The magnification of each figure is 6 x.

Fig. 6.3A Developing folds in the yolk sac of a 5 day old chick embryo. An arterial vessel (a) runs at the edge of the fold, which is still quite shallow. An intermediate vein (i) and a collateral vein (c) are also marked.

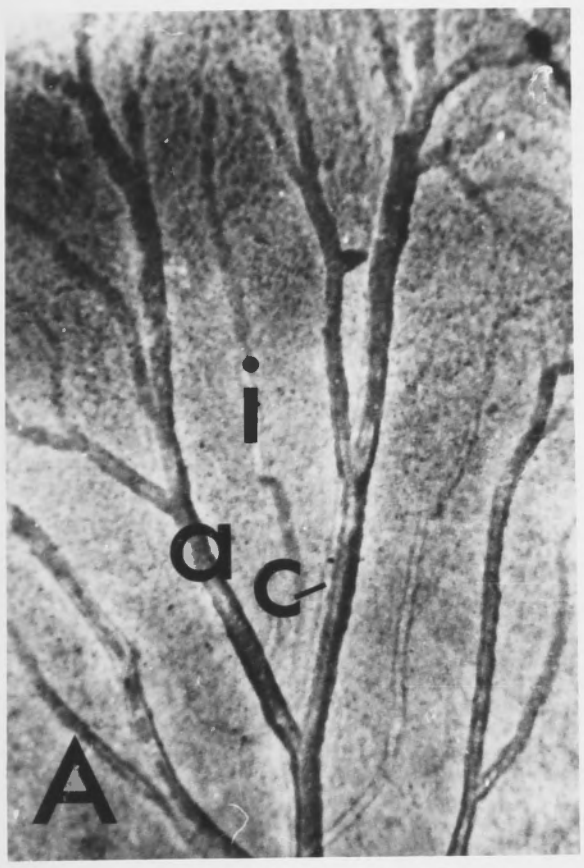
Fig. 6.3B 6 day yolk sac

Fig. 6.3C 7 day yolk sac

Fig. 6.3D 8 day yolk sac

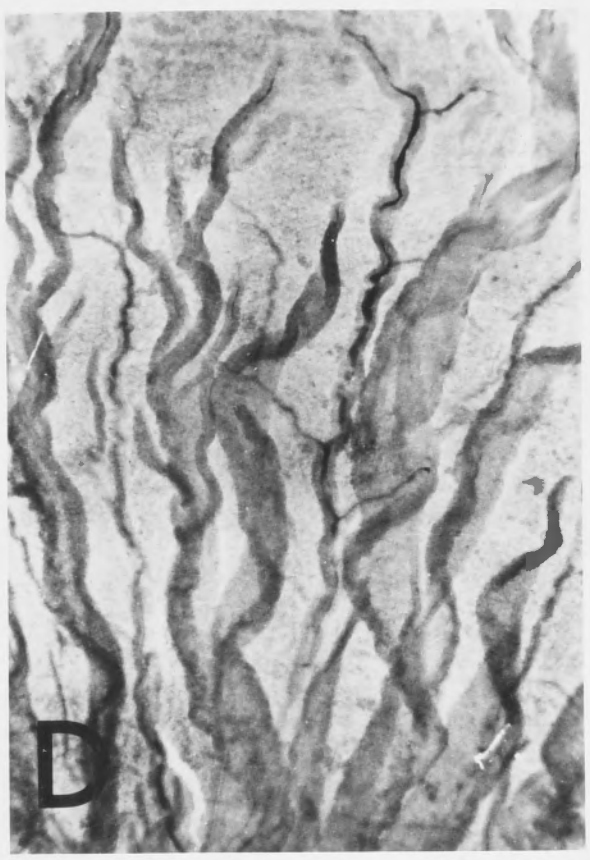
Fig. 6.3E Yolk sac fold (f) in a 10 day yolk sac. Between 5 to 10 days the yolk sac folds increase in height. A branch of the vitelline artery (a) runs near the margin of the fold.

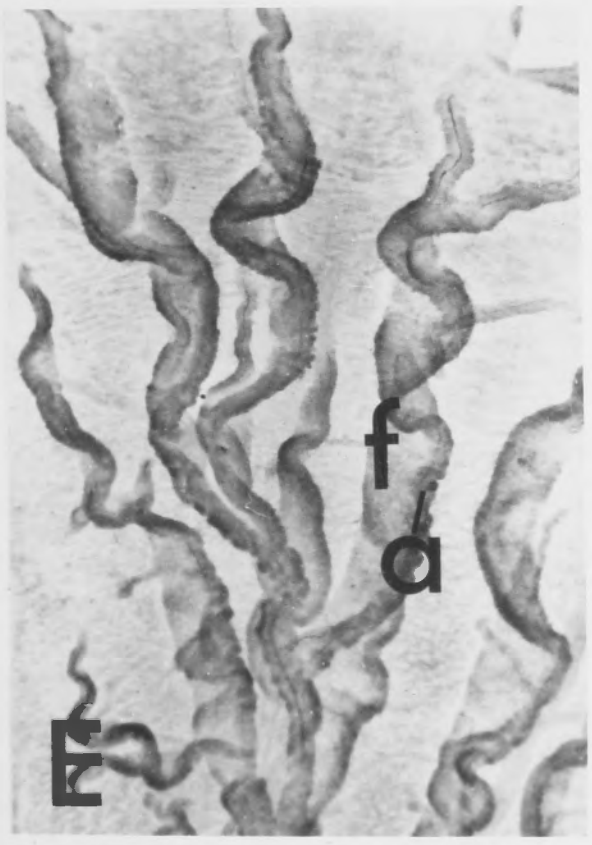
Fig. 6.3F Well developed yolk sac fold (f) from a 12 day yolk sac. The margin of the fold, which is moulded by the underlying vascular network, has become irregular in outline.


i
ac
A


B


C


D


f
a
E


f
F

capillaries of the superficial network are eventually drained by intermediate vessels running longitudinally between the yolk sac folds. At this stage, the collateral veins are very short and run for only a short distance along the proximal end of the yolk sac fold, draining blood from the intermediate veins into the lateral vitelline veins on either side of the embryo.

At 15 days the endodermal epithelium no longer forms a simple sheet covering the surface of the fold but has broken up into a lacy pattern of trabeculae oriented around the vessels of the venous plexus (fig. 6.4A).

In some animals which had been intravenously injected with carbon before the yolk sac was removed, superficial venous vessels and the vessels of the peri-arterial venous plexus showed an interesting carbon marking (fig. 6.4D). This finding was not examined further to determine whether this pattern was due to leakage from these vessels or possibly to intravascular sticking. Longitudinal sections through some of the deeper folds of the 15 day yolk sac often showed that they consisted of a series of long trabeculae from which the margin of the fold, containing the artery and the surrounding venous plexus, was suspended. This is illustrated in fig. 6.5A, a photomontage.

Relation of the arterial vessel to the capillary network of the fold

As pointed out previously, arteries running the length of yolk sac folds are said to have no communications with the plexus of small vessels surrounding them (Popoff, 1894). The validity of this statement was tested by following the artery and adjacent vessels through a sequence of serial sections and also by filling the yolk sac vasculature with two successive injections of differently coloured injection masses.

Three dimensional reconstruction of the blood vessels in a yolk sac fold

Serial sections were cut transversely through a fold from a 12 day yolk sac and outlines of the central artery and the surrounding venous capillaries were traced and then stacked between glass plates (chapter 2). One of these

Fig. 6.4A-C Yolk sac of a normal 15 day chick embryo injected with a carbon-coloured gelatine injection mass and cleared in glycerol to demonstrate the vascular system.

Fig. 6.4A Detail of a yolk sac fold, photographed as it projects into the yolk. An arterial vessel (a) follows the free edge of the fold and is surrounded by an intricate vascular network (n) from which longer and straighter vessels (l) communicate with the superficial capillary network (c) in the upper surface of the membrane. The superficial network is drained by intermediate veins (v) running between the yolk sac folds. A minor branch of the fold is shown at (bf).

Magnification 8 x.

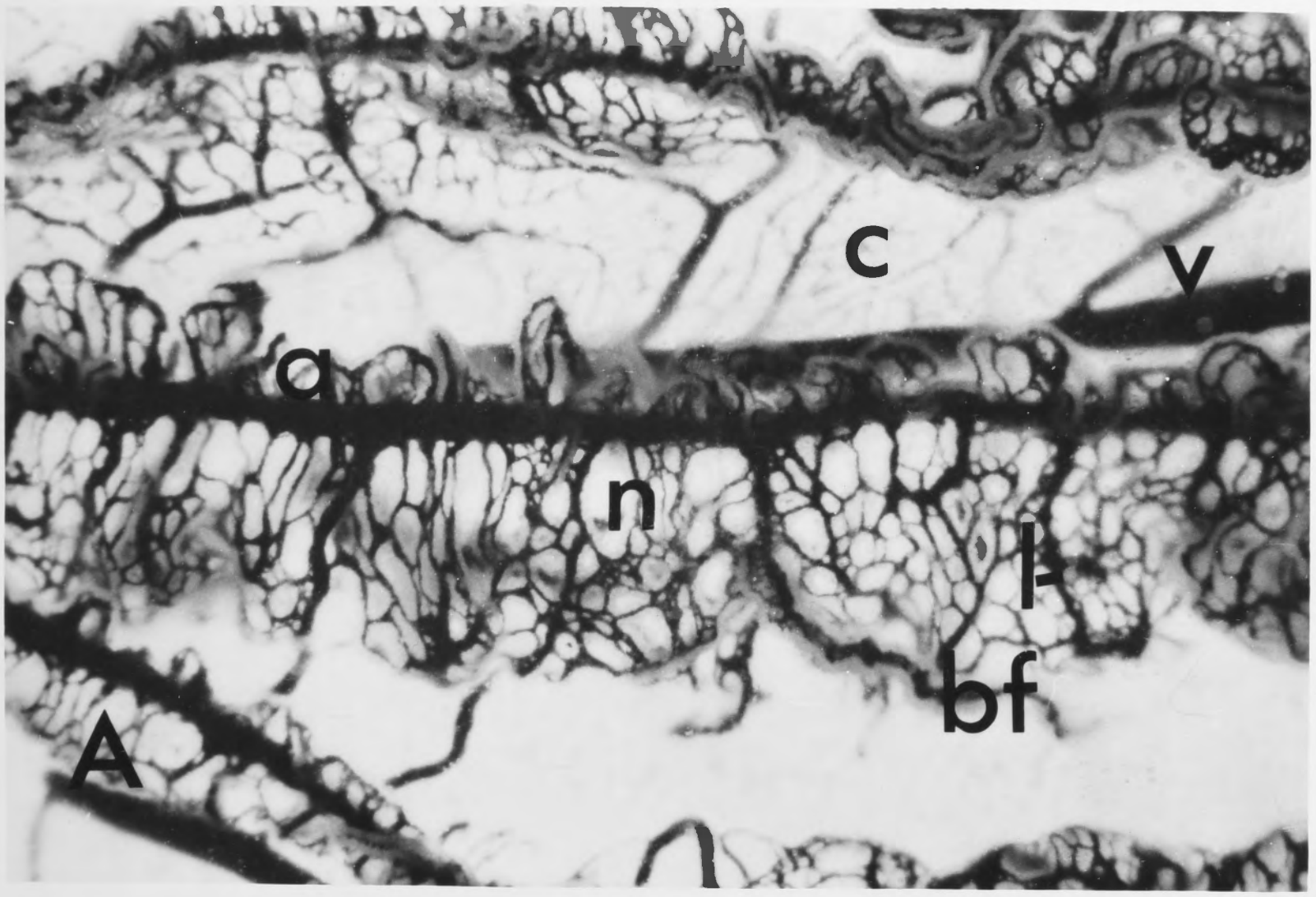
Fig. 6.4B Lower magnification of the same preparation, photographed from the yolk aspect. Fine terminal branches of folds and their arteries can be seen merging with the superficial capillaries and venous network (c) in the upper surface of the membrane. Intermediate veins (v) cross the field at the upper left and lower right.

Magnification 5 x.

Fig. 6.4C A similar field to fig. 6.4B, photographed from the upper surface to illustrate the superficial network (c) in relation to the folds (f) which appear as darker areas.

Magnification 5 x.

Fig. 6.4D Pattern of carbon marking in superficial venous vessels of the yolk sac of a 15 day chick embryo injected with colloidal carbon.


c
v
a
n
l
bf
A


v
f
c
B


c
v
f
C

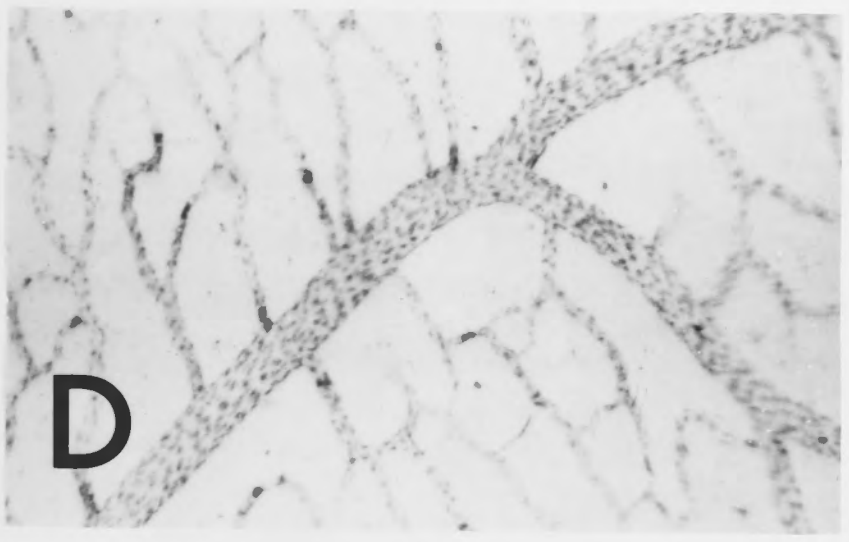
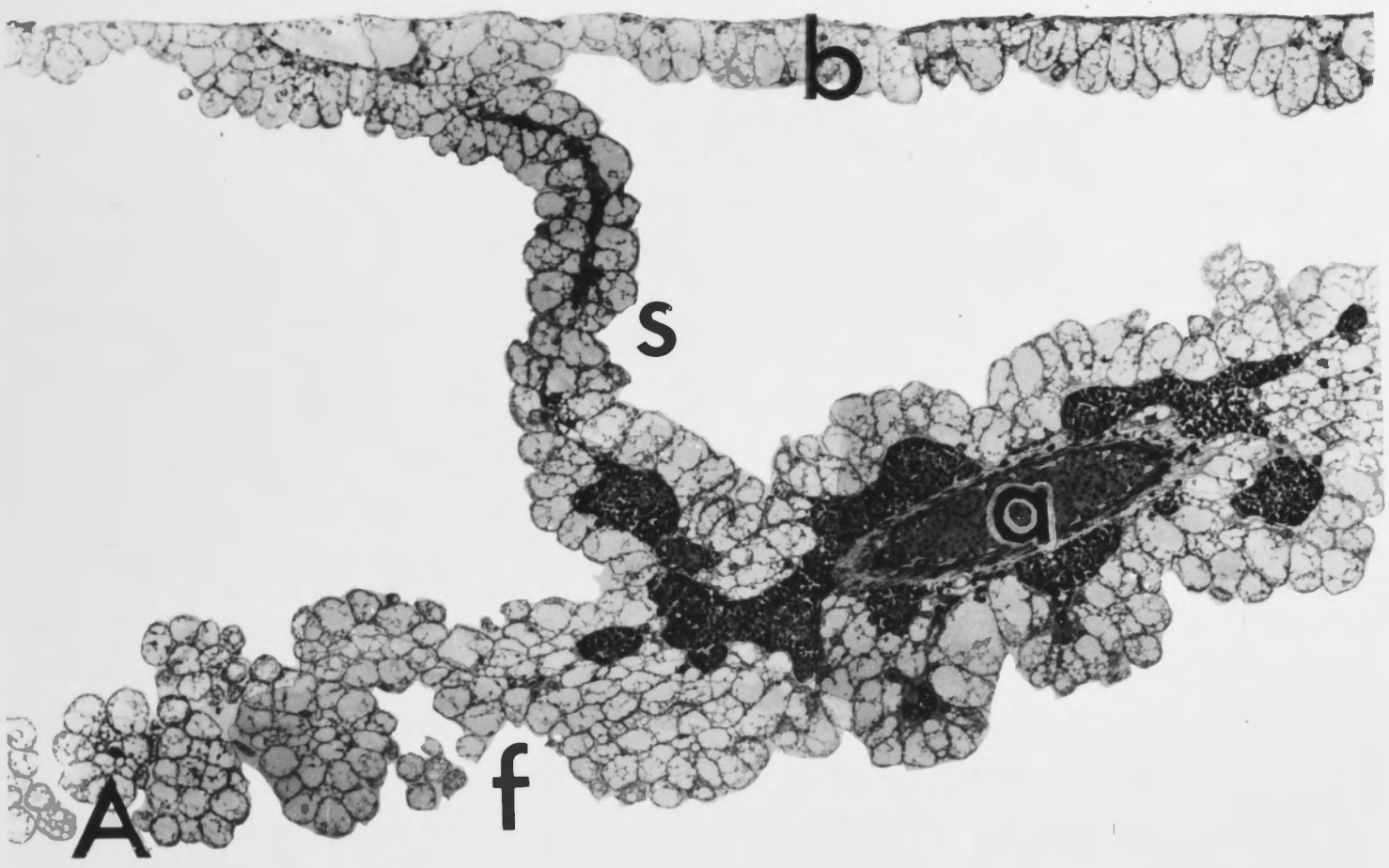

D

Fig. 6.5A Photomontage of an area from a longitudinal section through a yolk sac fold of a 15 day embryo. An epithelial cord (s) suspends the margin of the fold (f) containing an arterial vessel (a) and peri-arterial venous plexus. (b) marks the superficial surface of the yolk sac.
Azure II/methylene blue Magnification 90 x.

Fig. 6.5B The figure is taken from a sequence of serial sections across a yolk sac fold. The central artery (a) and venous capillary vessels of the peri-arterial plexus (c) are shown. The fold is covered with tall epithelial cells (e) containing numerous yolk inclusions.
Azure II/methylene blue Magnification 300 x.

Fig. 6.5C A three dimensional reconstruction of a segment of the yolk sac fold shown in fig. 6.5B. The central artery (a) does not communicate with the capillary vessels (c_1) but the merging of two vessels (c_1) and (c_2) of the surrounding network is shown.

Magnification 300 x.


b
s
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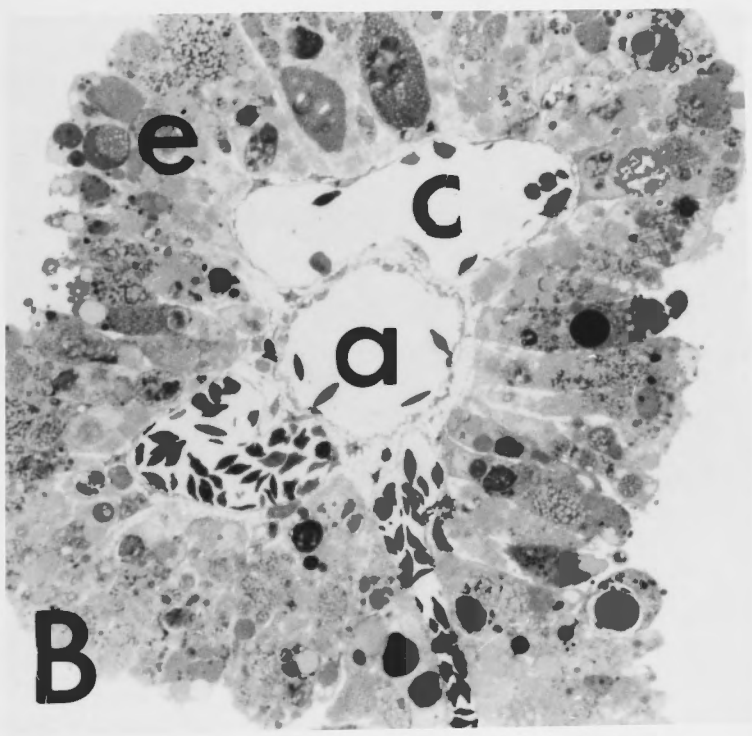
A
f


e
c
a
B


c
c
a
C

sections is shown in fig. 6.5B. The reconstruction represents a depth of approximately 100 μ in which connections occurred between adjacent capillary vessels (C_1 and C_2, fig. 6.5C) but were not observed between the capillary vessels and the central artery.

Since the sampling of relatively short segments of the fold, however, could easily miss arterial branches occurring at greater intervals, examinations were made of doubly injected whole mount preparations.

Topographic studies of blood vessels in the yolk sac folds

Yolk sac vessels of 15 day chick embryos were first filled with a carmine coloured gelatine mass and then over-injected with a small amount of carbon coloured gelatine to fill some of the arterial vessels. Topographical studies were made on unsectioned material mounted in glycerol jelly.

Part of a yolk sac which has been over-injected in this manner is illustrated in fig. 6.6. The capillary and venous network in the outer layer of the membrane and the capillary plexus within the yolk sac folds are filled with the carmine coloured gelatine injection mass. Some of the arterial vessels at the margin of the yolk sac folds, however, are filled with carbon coloured gelatine. At intervals along the fold these arteries give off small branches, also coloured with carbon, which join vessels in the peri-arterial venous plexus. It was concluded from these observations that the arterial vessel supplies the venous capillary network in the yolk sac folds.

Histological appearance of normal yolk sac folds

Yolk sacs from inbred CC chick embryos were sectioned along or across the yolk sac folds and examined in the light microscope. Although the folds increased in height and complexity from day 7 to day 12, there was little difference in the appearance of the peri-arterial venous vessels. Fig. 6.7A, a low power light micrograph, shows a cross section through a yolk sac fold of a 12 day embryo and fig. 6.7B enlarges the area around the central artery. Vessels of the peri-arterial plexus are the site of intense haemopoiesis. Large primitive stem cells (fig. 6.7C) crowd

Fig. 6.6 The yolk sac from a 15 day old chick embryo which was injected with a carmine coloured gelatine mass, over-injected with a small amount of carbon coloured gelatine and cleared in glycerol to illustrate the topography of the blood vessels in the yolk sac folds. The injection of carbon coloured gelatine was sufficient to fill the arterial vessels running at the margin of the yolk sac folds (a), which were found to give off branches at frequent intervals (b). These arterial branches are also coloured with carbon and join the network of venous capillaries surrounding the arterial vessel. Although most of these peri-arterial vessels are filled with the carmine coloured injection mass, in some areas, near an arterial branch, this network has also been over-injected with carbon (n).

Magnification 24 x.

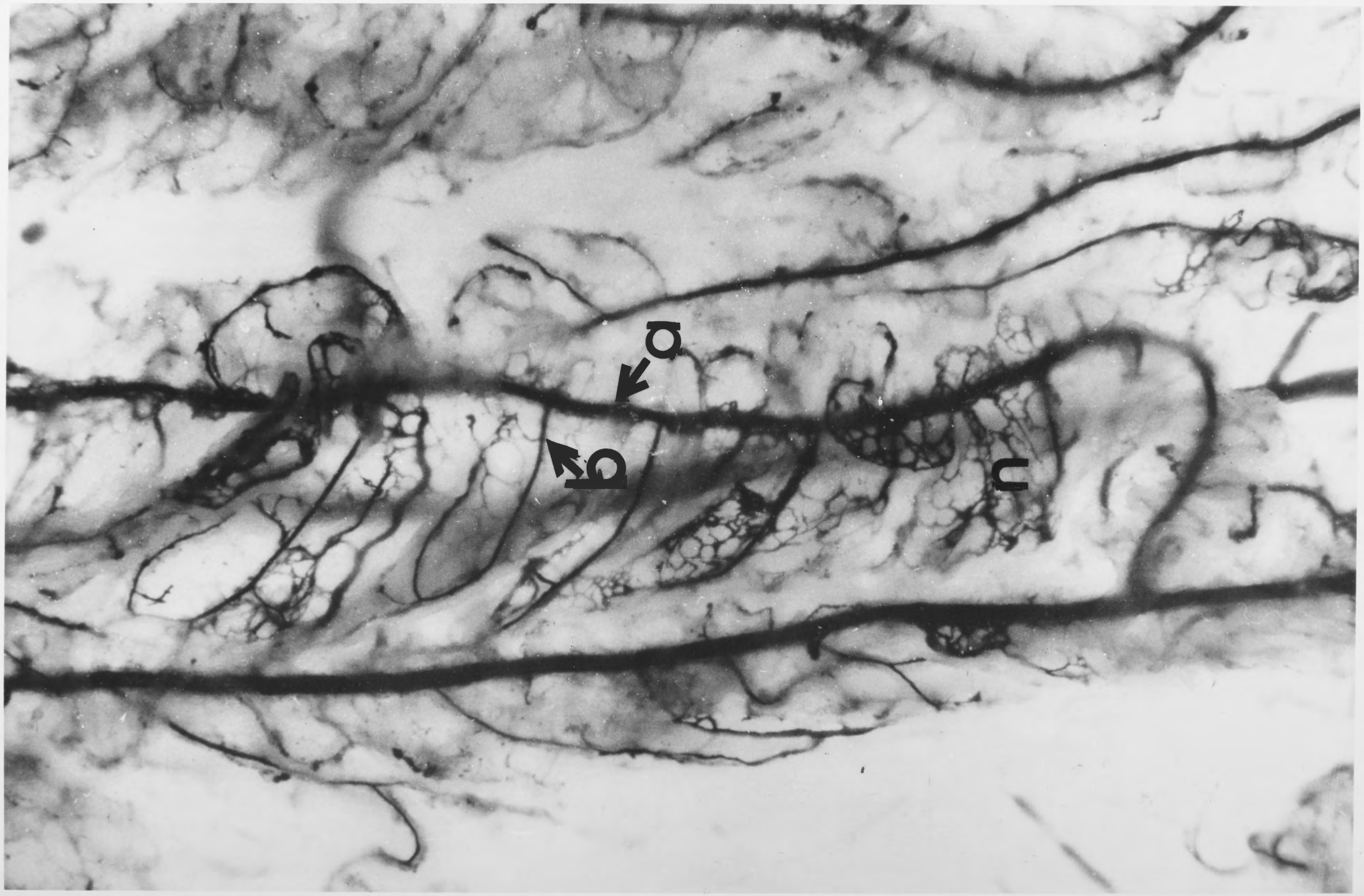
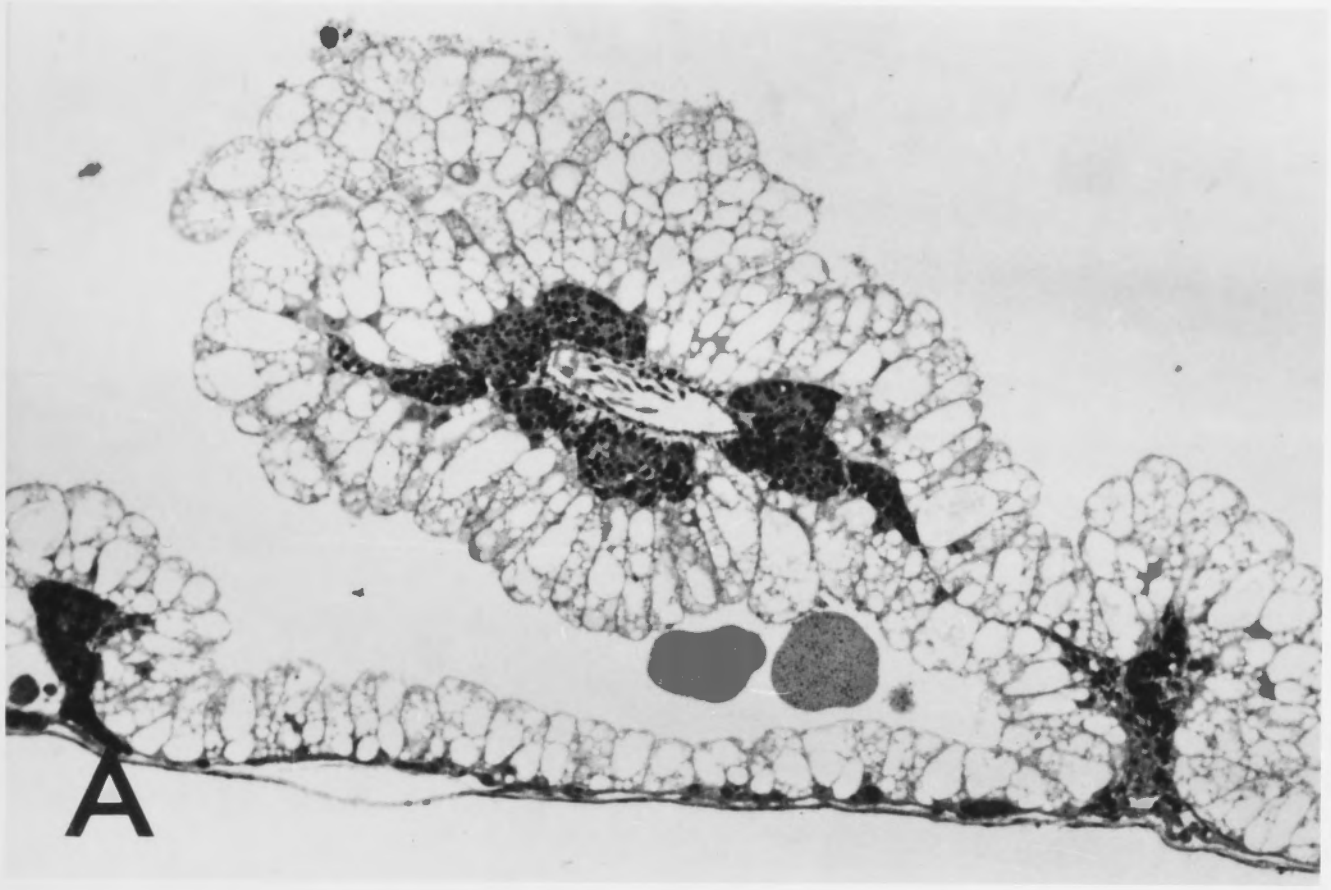

a
b
c

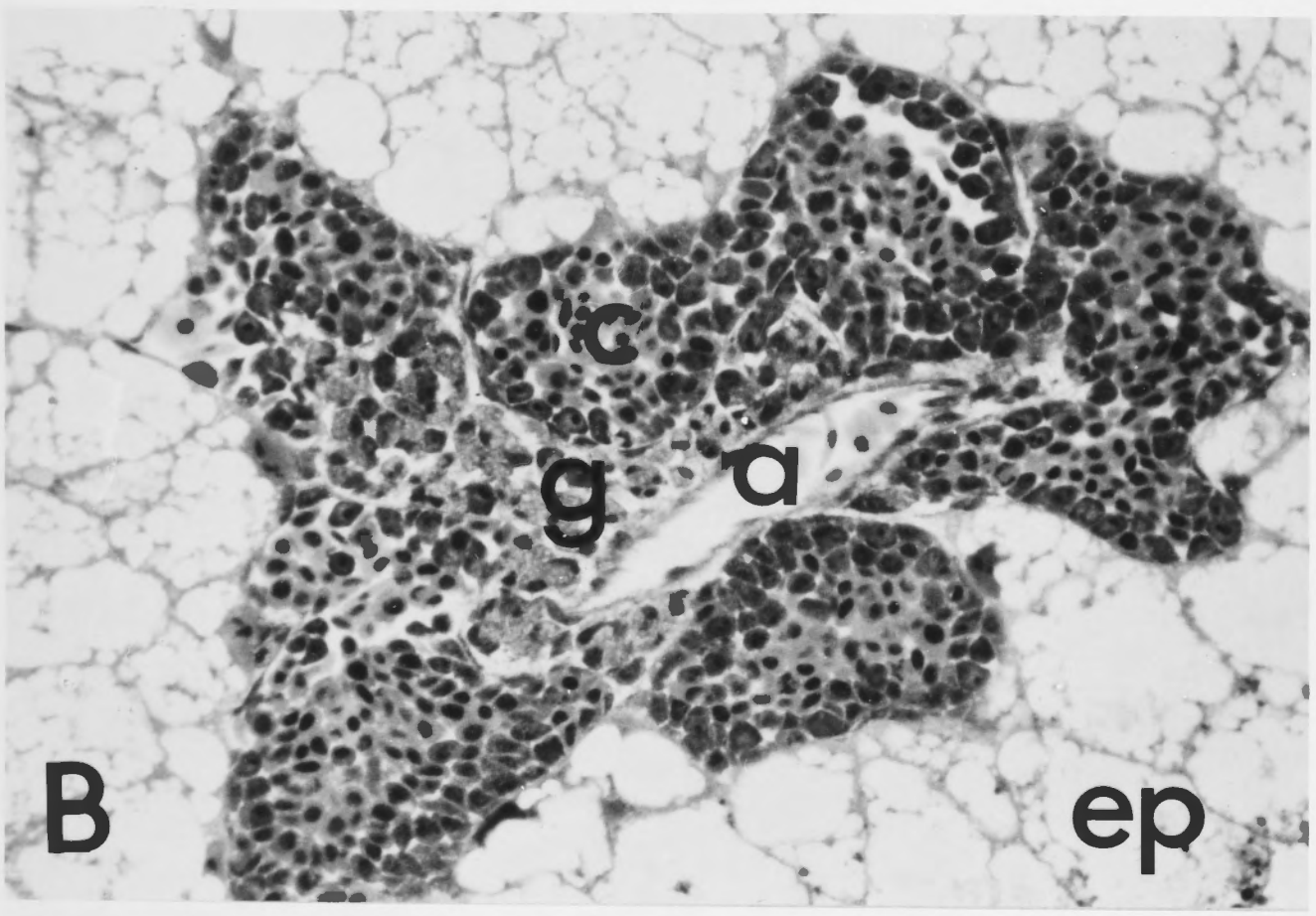
Fig. 6.7A-C Transverse sections of yolk sac folds from a normal 12 day chick embryo illustrating the normal structure of the fold and the blood islands.

Fig. 6.7A Section through a fold at a low magnification. Azure II/methylene blue Magnification 140 x.

Fig. 6.7B Higher magnification showing the central artery (a) surrounded by venous capillaries (c) of the peri-arterial plexus in which intense erythropoiesis is taking place. Granulocytic cells (g) develop around these vessels. The yolk sac epithelium is marked (ep).
Azure II/methylene blue Magnification 350 x.

Fig. 6.7C Detail of the blood islands. Haemopoietic stem cells (s) near the endothelium (e) give rise to erythrocytic progeny (m) towards the centre of the lumen. Granulocytic cells (g) develop in areas surrounding these vessels.
Azure II/methylene blue Magnification 1,400 x.


A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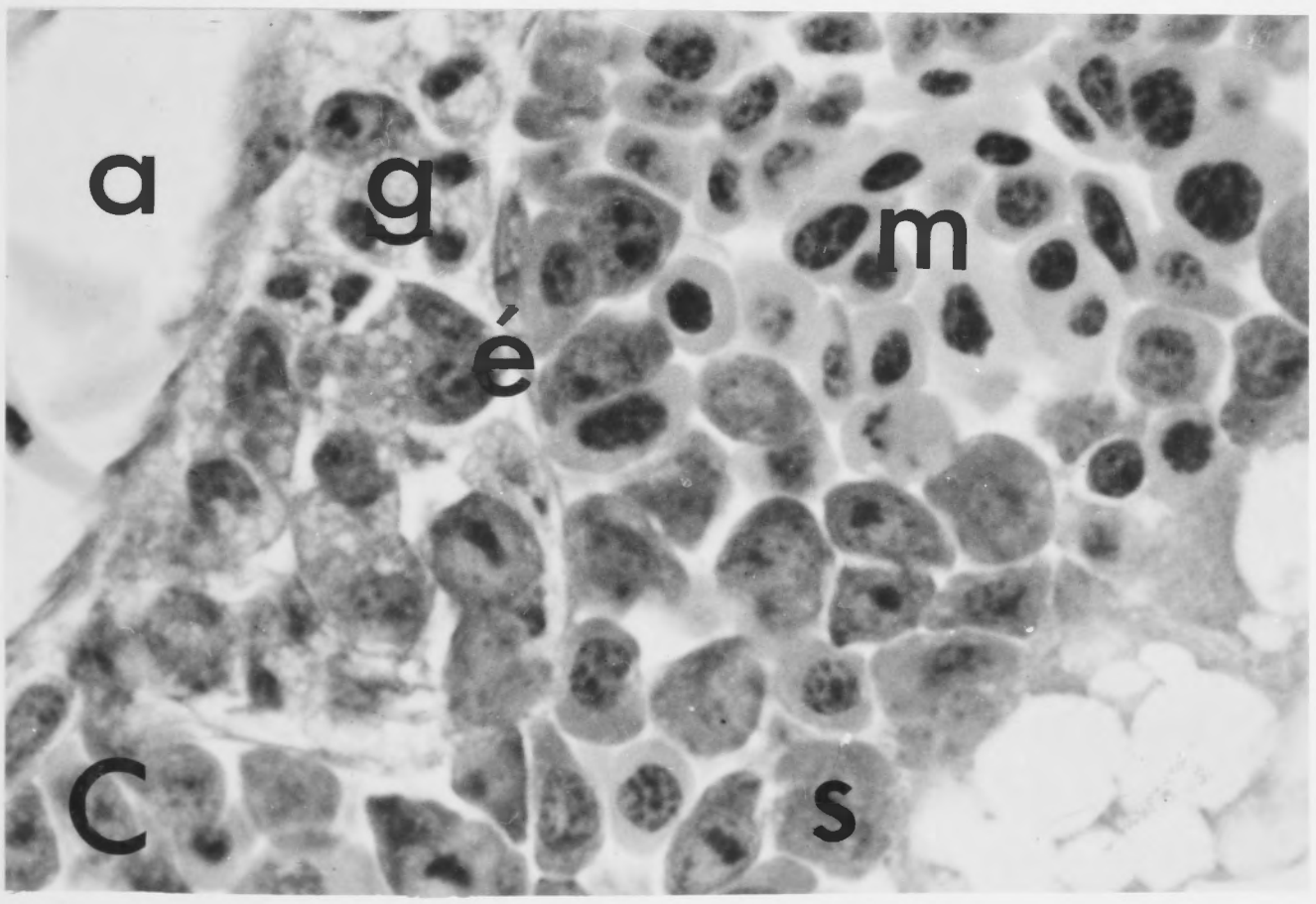
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against the tenuous endothelial lining. Towards the centre of the vessel, there are maturing red blood cells which are less tightly packed together (figs. 6.7B and 6.7C).

Granulopoiesis occurs extravascularly. Small groups of developing granulocytes commonly appear around these venous vessels either on the side facing the central artery as shown in fig. 6.7C or facing the yolk sac epithelium.

Gross and histological changes in the yolk sac during a GVHR

The effect of a GVHR on the yolk sac was examined in whole mounts and by light microscopy. Inbred CC embryos were used, inoculated on the CAM at 6 days with diluted adult AA blood. The yolk sacs were sampled 1 to 6 days after inoculation.

Three days after inoculation, most of the yolk sacs examined (8/10) were poorly developed. The folds were low and had few bifurcations. The yolk sac as a whole was more fragile than that of normal embryos of that age and often there was congestion of blood in the marginal vein. These changes were even more severe in embryos examined at later stages.

Histologically, changes were apparent even 1 day after the inoculation of allogeneic donor cells. In the yolk sac of most recipients examined (6/8) cells in the blood islands appeared depleted. This is evident in fig. 6.8A as compared to fig. 6.7B. Blood cells were scattered loosely throughout the lumen of the vessel and some had ragged or crenulated outlines and appeared to be degenerating. Clumps of cell debris were also seen among the intact cells.

Two days after inoculation depletion of haemopoietic stem cells in the blood islands was even greater in many (6/10) of the yolk sacs examined (fig. 6.8B and C). Few haemopoietic cells of any kind remained within the venous capillary vessels. The surrounding yolk sac epithelium, however, appeared normal. Fig. 6.8C shows that the endothelial lining of the venous vessels remains intact and the remaining intravascular cells are scattered along the wall of the vessel either singly, or in small clusters; they include both primitive, darkly staining, haemopoietic stem cells and more mature cell elements. Granulocytic cells were not seen. In 4 of the 10 embryos examined at this

Fig. 6.8A-E Sections from the yolk sac of chick embryos which have been inoculated on the CAM at 6 days with 0.1 ml of a 1:1 dilution of adult AA blood in Alsever's solution. The figures show the changes which occur in the blood islands from 1 to 4 days after inoculation. The following features are marked : the central artery (a) surrounded by venous capillaries (c) which contain haemopoietic stem cells (h) and developing red blood cells (r); the yolk sac epithelium (e) and the epithelial cell nuclei (n) and clumps of cell debris (cd). All sections are stained with azure II/methylene blue.

Fig. 6.8A One day after the inoculation of adult allogeneic blood the blood islands (c) around the central artery (a) of the fold have already become depleted.

Magnification 400 x.

Fig. 6.8B Two days after inoculation former haemopoietic areas surrounding the artery (a) of a fold are nearly empty.

Magnification 120 x.

Fig. 6.8C Detail from a field such as shown in fig. 6.8B. The endothelial lining appears uninterrupted. Only a few haemopoietic cells (h) adhere to the endothelium.

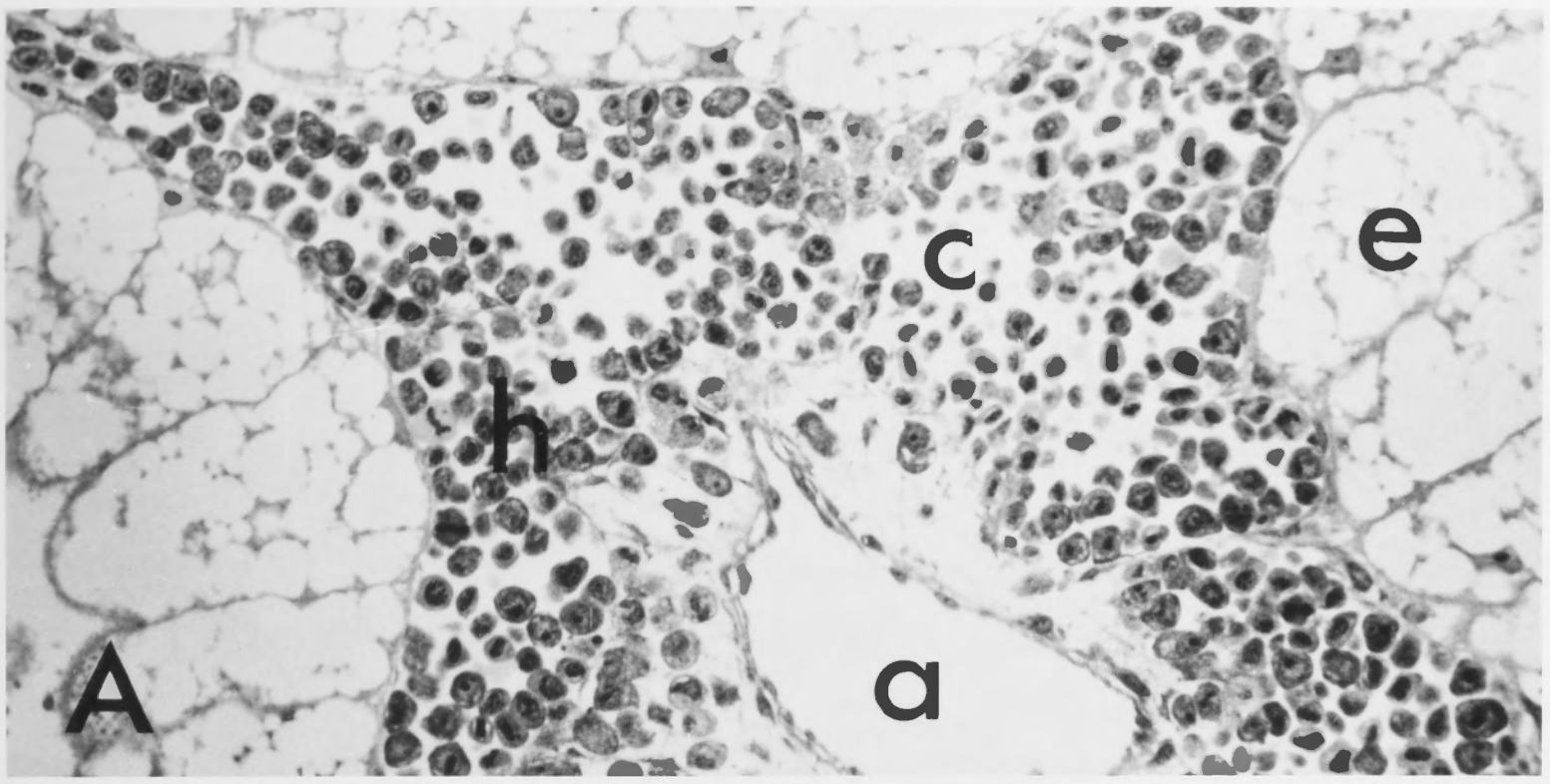
Magnification 1,200 x.

Fig. 6.8D Three days after inoculation more of the lumen is occupied by clusters of degenerating cells.

Magnification 1,200 x.

Fig. 6.8E A similar field 4 days after inoculation. Only clumps of cell debris remain within the lumen of the peri-arterial venous capillaries.

Magnification 1,200 x.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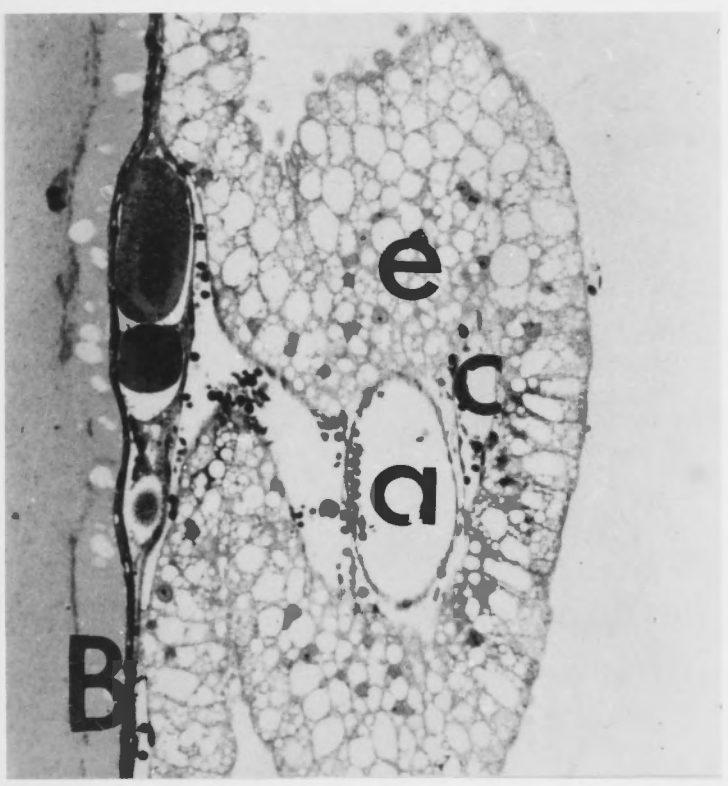
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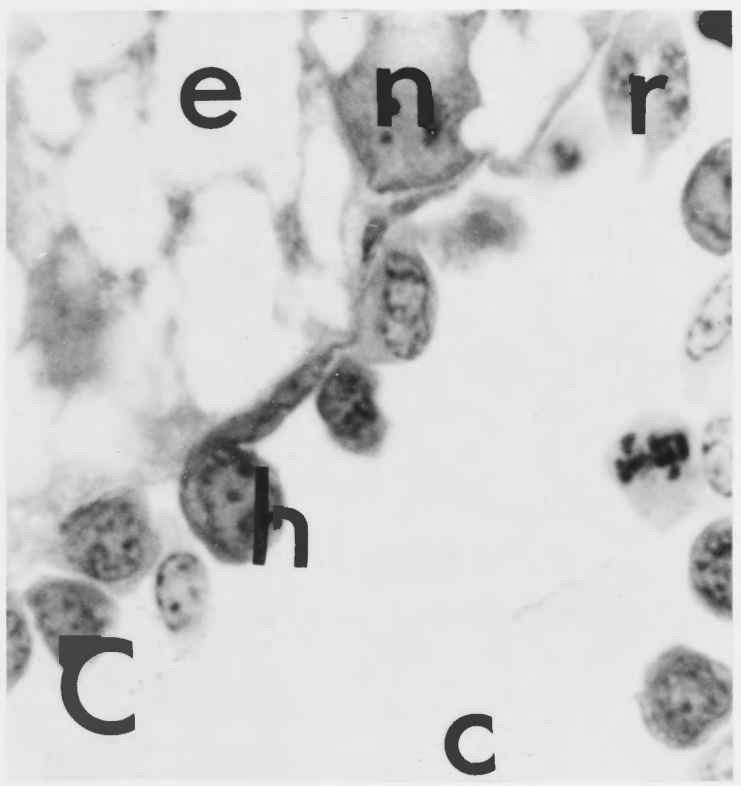
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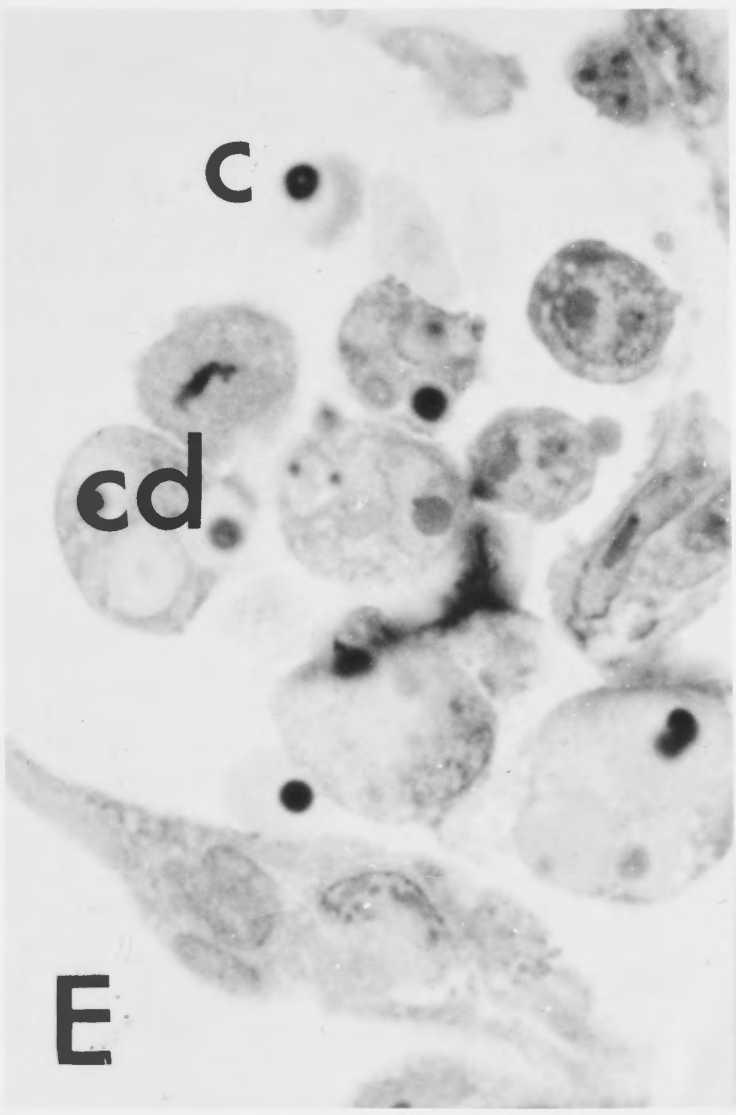
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stage small haemorrhages of the type described in chapter 4 occurred.

By 3 and 4 days after the inoculation two-thirds of the embryos (8/12 in each group) had died, presumably as a result of the GVHR. In the surviving embryos, the blood islands of the yolk sac were severely depleted. Very few haemopoietic cells could be found which still appeared to be viable. Instead, large clusters of degenerating cells, such as those shown in figs. 6.8D and E were present within the venous capillary vessels of the yolk sac fold.

Inoculation of adult allogeneic blood thus causes an almost total depletion of haemopoietic stem cells and their more differentiated derivatives, within the blood islands of the yolk sac within 3 or 4 days after the inoculation of adult allogeneic blood into the 6 day old embryo.

Fine structure of vessels in the yolk sac folds of 7 day normal chick embryos

Some observations were also made at the level of the electron microscope. Fig. 6.9A shows the appearance of a normal peri-arterial venous vessel in the yolk sac fold from a normal 7 day old chick embryo. The lumen of the vessel is crowded with developing haemopoietic cells, the most primitive being found at the periphery of the vessel closely apposed to the vascular endothelium. These are large spherical cells, with an abundant cytoplasm packed with many free ribosomes. Rod-shaped or oval mitochondria are numerous and there is a well defined golgi zone but very little endoplasmic reticulum. Primitive erythroid cells occur further towards the centre of the lumen. They have an ovoid rather than a spherical shape. Their cytoplasm has a greater density than that of the more primitive stem cells and also contains a large number of ribosomes but very little endoplasmic reticulum.

The venous capillaries in the fold are lined by elongate endothelial cells with an underlying basement membrane. Cells of the yolk sac epithelium may be closely apposed to the wall of the venous capillary (fig. 6.9A) or they may be separated by clusters of developing granulocytic cells as shown in fig. 6.9B. The nucleus of the epithelial cells is usually in the basal area of the cell and is surrounded by

Fig. 6.9A Electron micrograph illustrating the normal structure of a venous capillary vessel in the yolk sac fold of a 7 day old CC embryo. The vessel is thin walled and primitive haemopoietic cells (ph) are closely apposed to the vessel endothelium (e). The cytoplasm (c) of these primitive haemopoietic cells contains many free ribosomes, tubular mitochondria (mt), a golgi apparatus (g) but little endoplasmic reticulum. Developing red cells (dr) occur further towards the centre of the lumen. An epithelial cell (ye) with a large cell nucleus (n) is shown on the left.

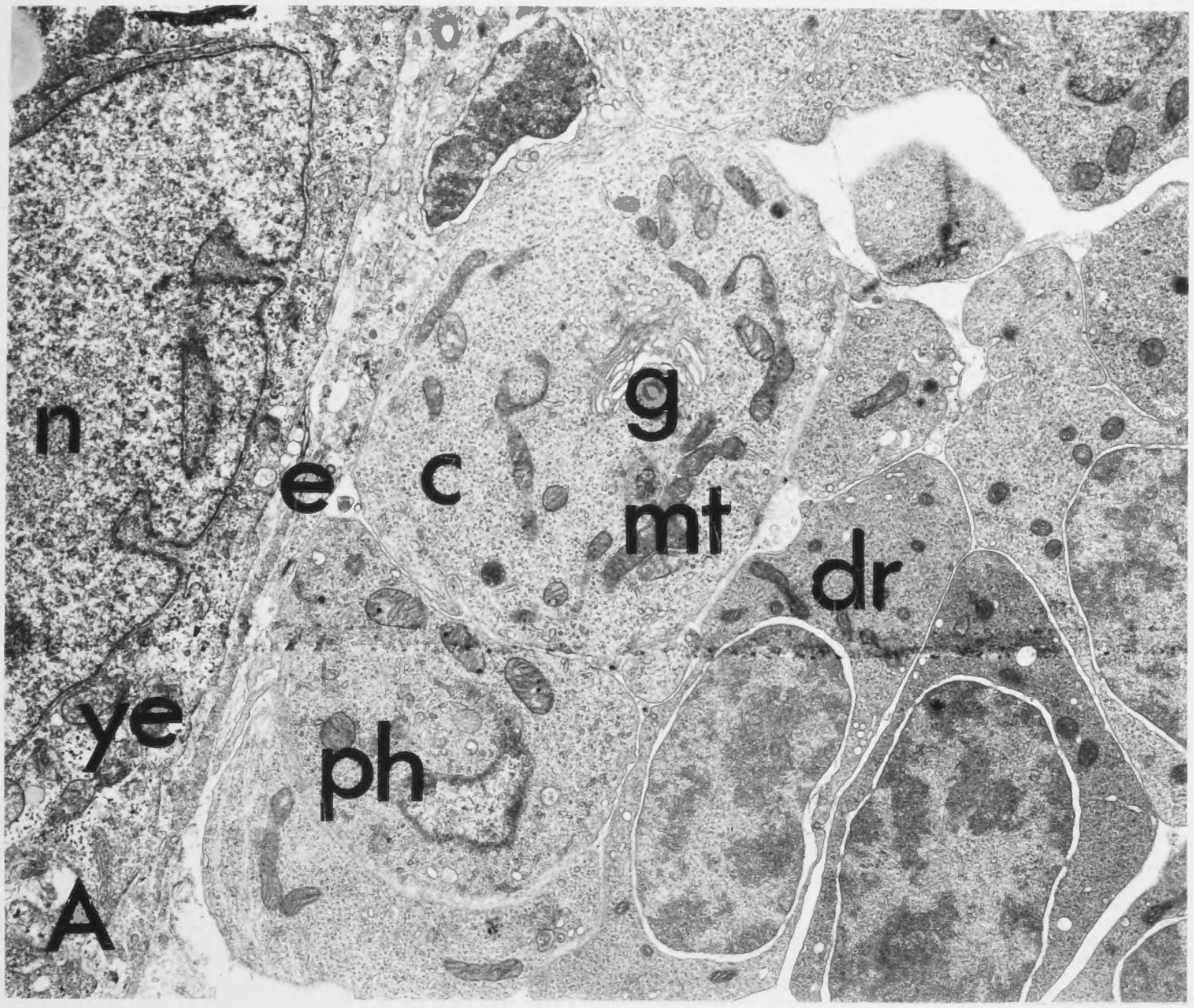
Magnification 10,000 x.

Fig. 6.9B Developing granulocytic cells (gr) and their precursors (gp) outside the endothelium (e) of the peri-arterial venous capillary vessel from a yolk sac fold of a 7 day embryo. A developing red cell (dr) lies within the lumen of the vessel.

Magnification 10,000 x.

Fig. 6.9C Venous capillary vessel from a yolk sac fold of a 7 day CC chick embryo which has been inoculated on the CAM at 6 days with diluted AA adult blood. Degenerating cells are seen in the lumen. The cell marked (d) resembles a thrombo-cyte. The endothelium (e) of these vessels is intact al-though vacuolization is apparent. Collagen fibres (cf) and fibroblasts (fb) are also marked.

Magnification 10,000 x.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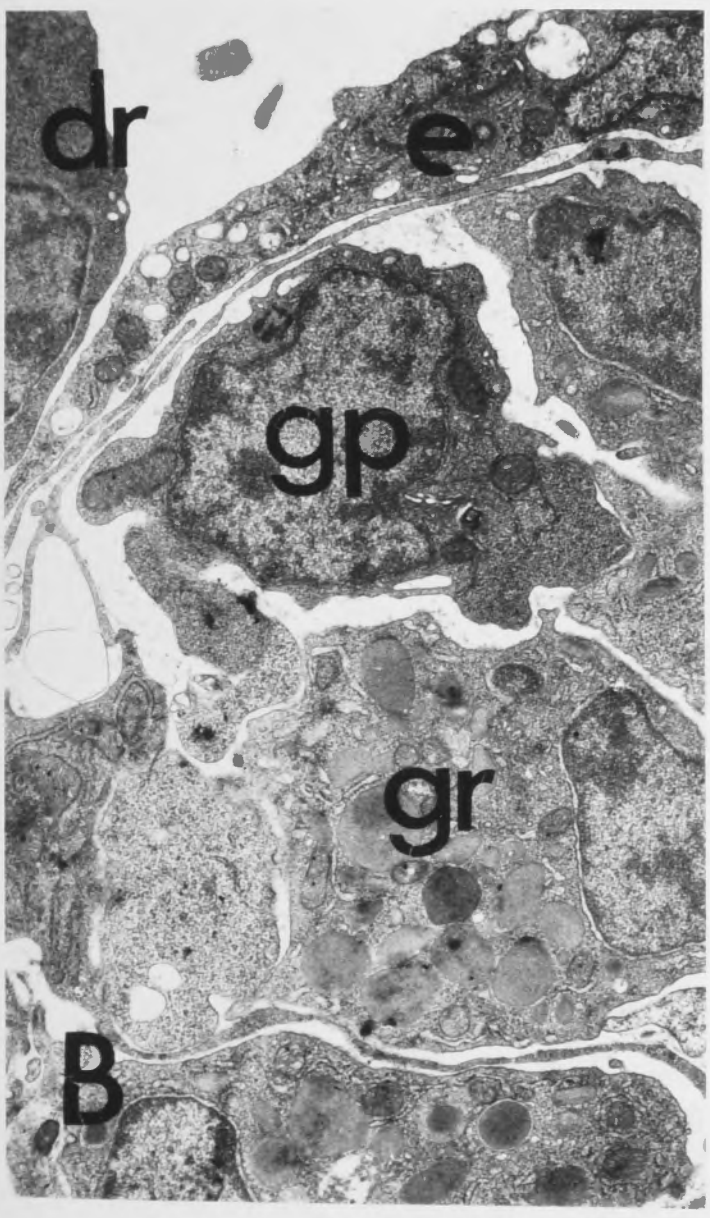
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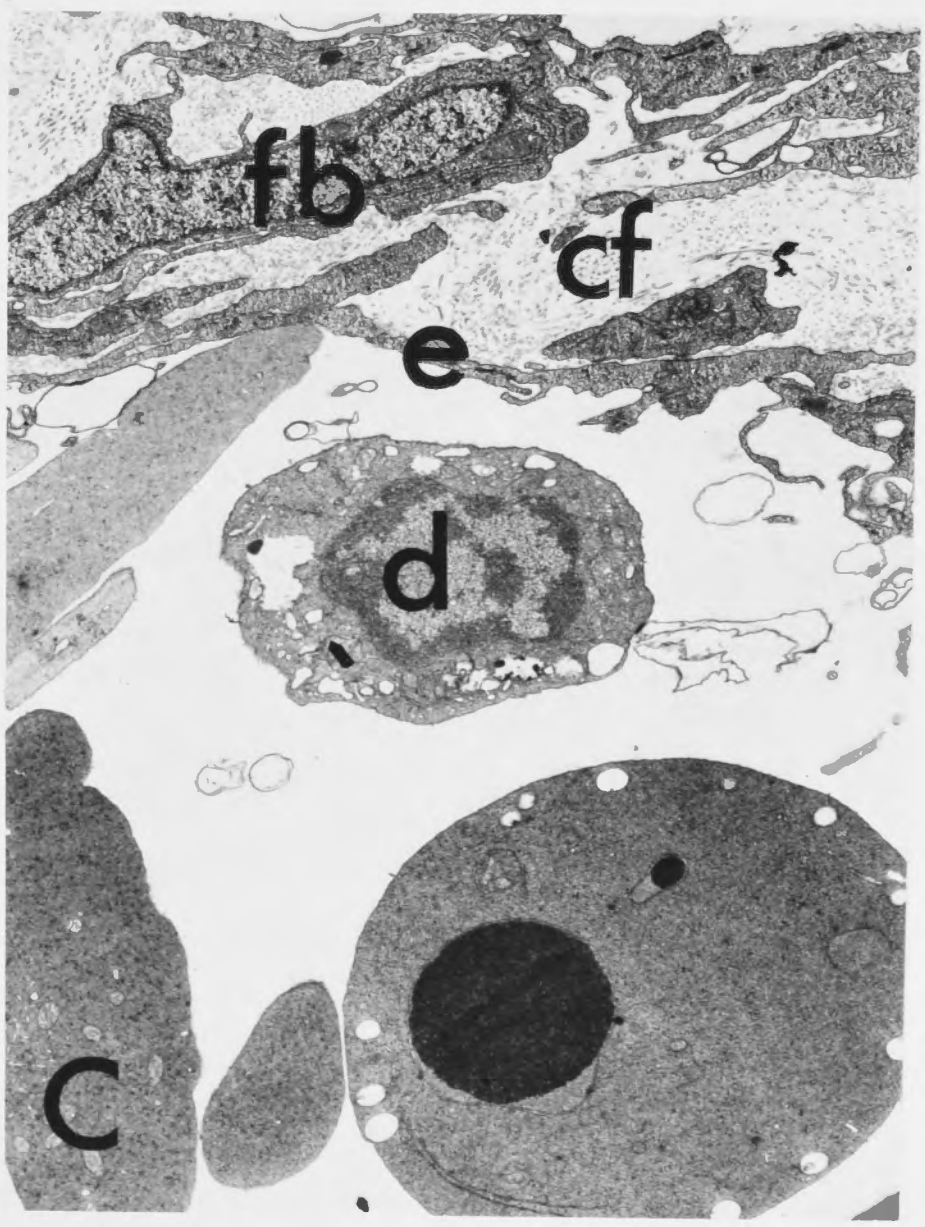
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cytoplasm which contains many spherical yolk inclusions.

Clusters of developing granulocytes (fig. 6.9B) include both primitive and more mature cells. The primitive cells have a dense cytoplasm containing many free ribosomes and little endoplasmic reticulum. The more mature cells, however, have many large, spherical or oval electron dense granules.

Changes in fine structure in the peri-arterial venous vessels in the yolk sac folds of chick embryos undergoing a GVHR

The yolk sac from inbred CC chick embryos inoculated with allogeneic blood at day 6 was examined with the electron microscope 1 day after inoculation. Changes which had occurred are similar to those which have already been described by light microscopy.

Few haemopoietic stem cells remain in the peri-arterial venous capillary vessels (fig. 6.9C) and those which are left appear to be almost mature erythroid elements. Many of them show degenerative changes, especially vacuolation. While in most cases, the endothelial layer of these vessels appeared to be undamaged, in some areas highly vacuolated endothelial cells were also seen.

Although only a few electron microscopic observations were made in embryos undergoing a GVHR, they confirmed observations already described at the level of the light microscope. The major change was the severe depletion of haemopoietic cells of the blood islands in the yolk sac folds.

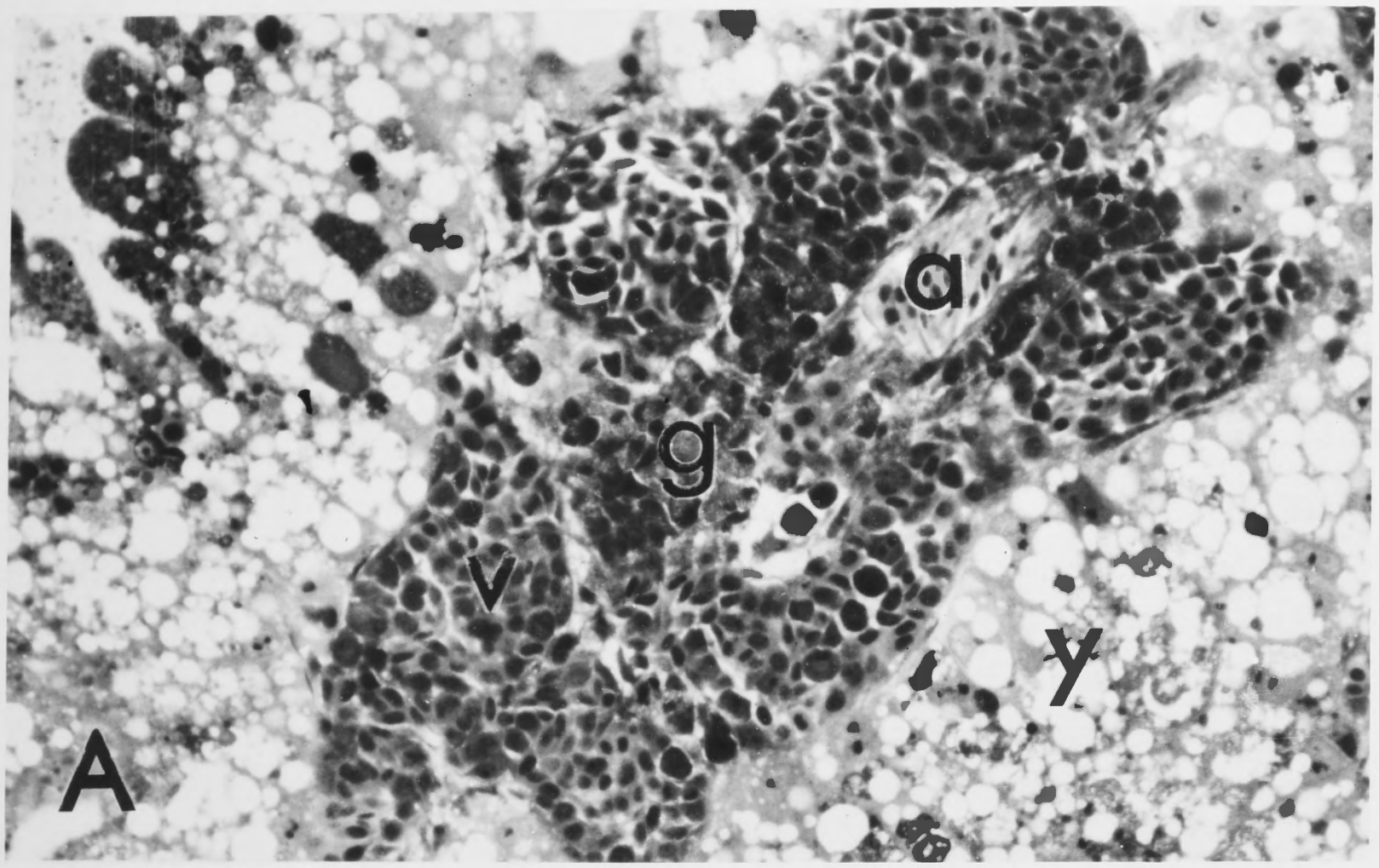
The specificity of the haemopoietic cell depletion in the yolk sac folds

In order to determine whether depletion of haemopoietic cells in the blood islands of the yolk sac fold was specifically due to the development of a GVHR, some animals were examined which had been inoculated with syngeneic cells. Six days after the inoculation of adult AA blood diluted 1:1 in Alsever's solution into 6 day old AA embryos, the blood islands in the yolk sac folds were entirely normal in appearance. As shown in figs. 6.10A and B, no depletion of haemopoietic cells had occurred. Depletion of haemopoietic cells within the blood islands of the yolk sac is thus a

Fig. 6.10A-B Sections from the yolk sac of a 12 day old AA chick embryo inoculated at 6 days with 0.1 ml of a 1:1 dilution of adult AA blood in Alsever's solution. As seen in these sections, the inoculation of adult syngeneic blood has no effect on the development of the blood islands.

Fig. 6.10A No evidence of cell depletion can be found in the venous capillary vessels (v) around the central artery (a) which are crowded with haemopoietic stem cells and their more differentiated derivatives. Granulocytic cells (g) are seen in clusters outside the vascular lumen. The yolk sac epithelium (y) also appears normal.
Azure II/methylene blue Magnification 400 x.

Fig. 6.10B At high magnification many developing red cells (dr) can be seen within the peri-arterial venous capillaries. Granulocytic cells (g) differentiate within the perivascular space between the venous capillaries and the arterial vessel (a).
Azure II/methylene blue Magnification 1,600 x.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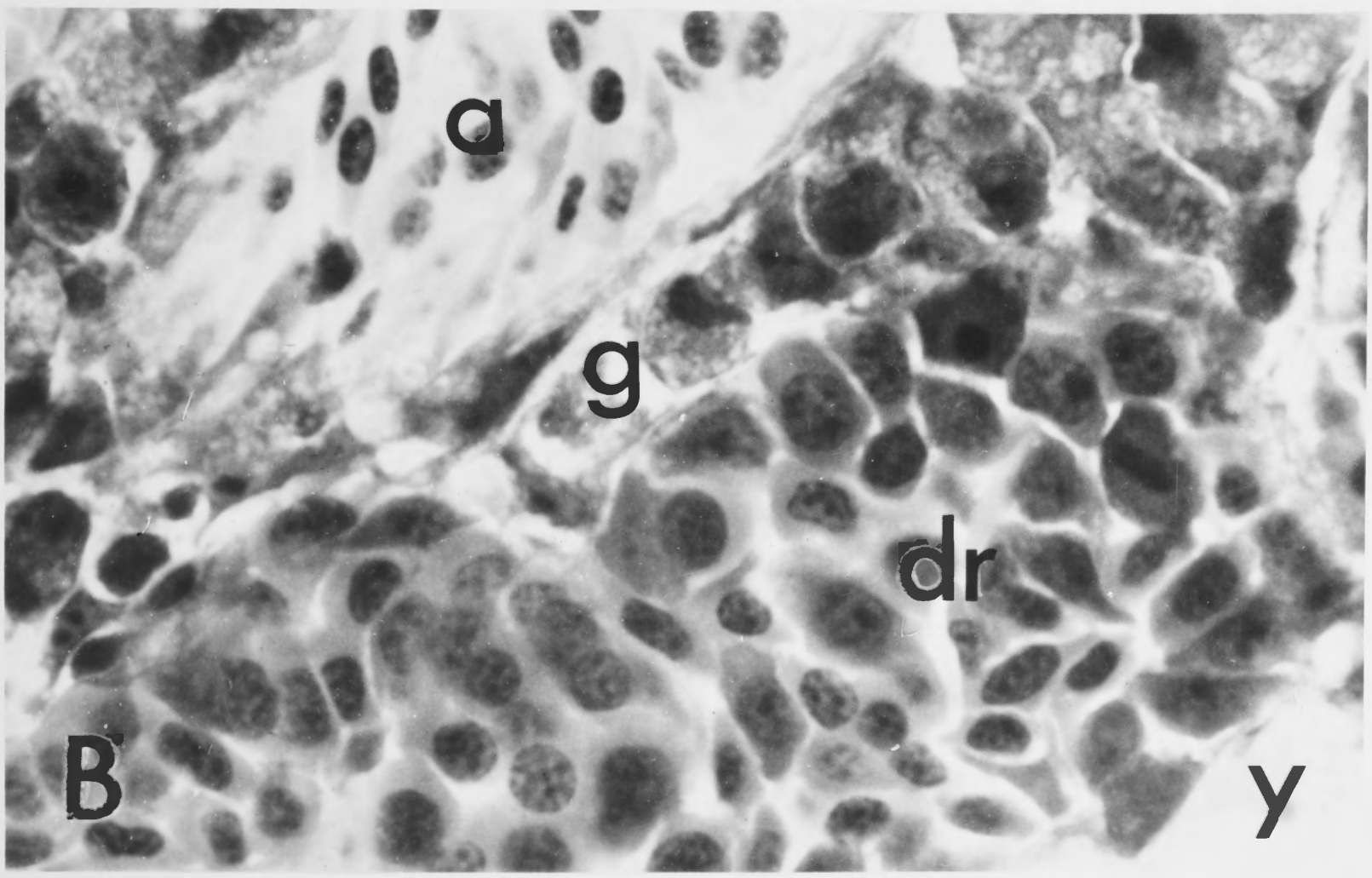
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specific pathological change which only occurs after the inoculation of adult allogeneic blood into the developing chick embryo.

Discussion

Popoff (1894), who studied carbon injected yolk sac preparations, had described the circulatory system within a yolk sac fold from a 20 day embryo. He found that the capillary network within the fold drained into 2 long capillary vessels which ran parallel to the arterial vessel on each side of the fold. Venous vessels of this type, however, were not seen in the present study.

Popoff also considered that the arterial vessel in the yolk sac fold did not communicate with the peri-arterial venous plexus which surrounded it. Double injection of yolk sacs has shown, however, that this is not the case, since branches from the arterial vessel were demonstrated which join the peri-arterial venous plexus.

The inoculation of adult allogeneic blood into 6 day old CC chick embryos caused severe cell depletion in the blood islands of the yolk sac fold. It seems likely that this cell damage may contribute to the increased lysosomal enzyme content of the plasma, described in chapter 5. Since haemopoietic stem cells in the yolk sac are known to give rise to thrombocytes (Sugiyama, 1926; Edmonds, 1966), destruction of yolk sac stem cells may also produce thrombocytopenia, a condition which may explain the development of haemorrhages during a GVHR in very young chick embryos as discussed in chapter 4. The development of thrombocytopenia in 20 day old chick embryos inoculated at day 10 with adult blood has been reported by Macpherson and Deamer (1965).

Since the depletion of stem cells in the blood islands did not occur after the inoculation of syngeneic adult blood, this change may be specifically attributed to the development of a GVHR. It is not, however, a GVHR characterised by proliferative lesions as previously described (Simonsen, 1962) but one in which the donor cells destroy or inactivate haemopoietic stem cells in the blood islands of the host. There is some evidence that adult lymphocytes

may react against allogeneic haemopoietic stem cells in this manner in other animals.

Davis and coworkers (1969, 1970) used the spleen colony assay for haemopoietic stem cells developed for mice by Till and McCulloch (1961) to show that a decrease in the number of colony forming units occurred in the spleen and marrow of F_1 hybrid mice, following the injection of parental strain lymphoid cells. They therefore concluded (Davis et al., 1970) that colony forming cells may be a primary target during a GVHR.

Adult lymphocytes have also been shown to destroy allogeneic colony forming units in a situation where 2 grafted cell populations interact (Petrov et al., 1969, 1970). When lymph node cells from 1 parental strain were mixed with spleen or bone marrow cells from the other parental strain and inoculated into lethally irradiated F_1 recipients (Petrov et al., 1969) there was a significant or complete inactivation of colony forming units in the graft. Plaque-forming cells in the graft were also inactivated.

The haemopoietic stem cells which are responsible for spleen colony formation in the mouse are pluripotential stem cells (McCulloch and Till, 1970) which give rise to both erythrocytic and granulocytic progeny (Wu et al., 1967, 1968a). Lymphoid cells either descend directly from colony forming cells, or both cell classes have a common progenitor (Wu et al., 1968b). Thus, if colony forming cells are inactivated during a GVHR, erythropoiesis should be affected as well as granulopoiesis and lymphopoiesis.

There is some evidence that this is the case. Pinno and coworkers (1969) have shown that there is a sharp decrease in the erythropoietic activity of the bone marrow of F_1 hybrid mice injected with parental strain lymphoid cells. It has also been shown by Cornelius and coworkers (1969) that the development of a GVHR in F_1 hybrid mice lowers the reticulocyte response of the recipient to anaemia induced by daily bleeding.

The early experiments of Block (1946) on grafts of rat yolk sac may demonstrate a similar phenomenon. Pieces of yolk sac from 11 to 17 day rat embryos were grafted into the

anterior eye chamber of adult rats where they became vascularised within 4 days. In the normal rat yolk sac at this stage venous sinuses are filled with developing haemopoietic cells. In the grafted yolk sac, however, there was a sudden and complete disappearance of primitive erythroblasts while lymphocytes migrated into the graft from the host.

Although pluripotential stem cells similar to those which form spleen colonies in the mouse have been shown to occur in rats (Nowell et al., 1970) and humans (Senn and McCulloch, 1970), an assay for the detection of these cells has not yet been developed in the chick embryo. In the mouse embryo, the yolk sac has been shown to be the first organ in ontogeny to develop colony forming cells (Moore and Metcalfe, 1970). It would seem likely that an analogous cell type is present in the yolk sac of the developing chick embryo (Moore and Owen, 1967c). If this is the case, the cell depletion which occurs in the yolk sac during the GVHR may be the result of inactivation or destruction of primitive stem cells in a manner which is similar to the depression of colony forming units in the haemopoietic tissue of mice undergoing a GVHR (Davis et al., 1970).

CHAPTER 7

CHANGES IN THE DEVELOPMENT OF EMBRYONIC HAEMOPOIETIC TISSUE DURING A GRAFT-VERSUS-HOST REACTION : BURSA, THYMUS AND BONE MARROW

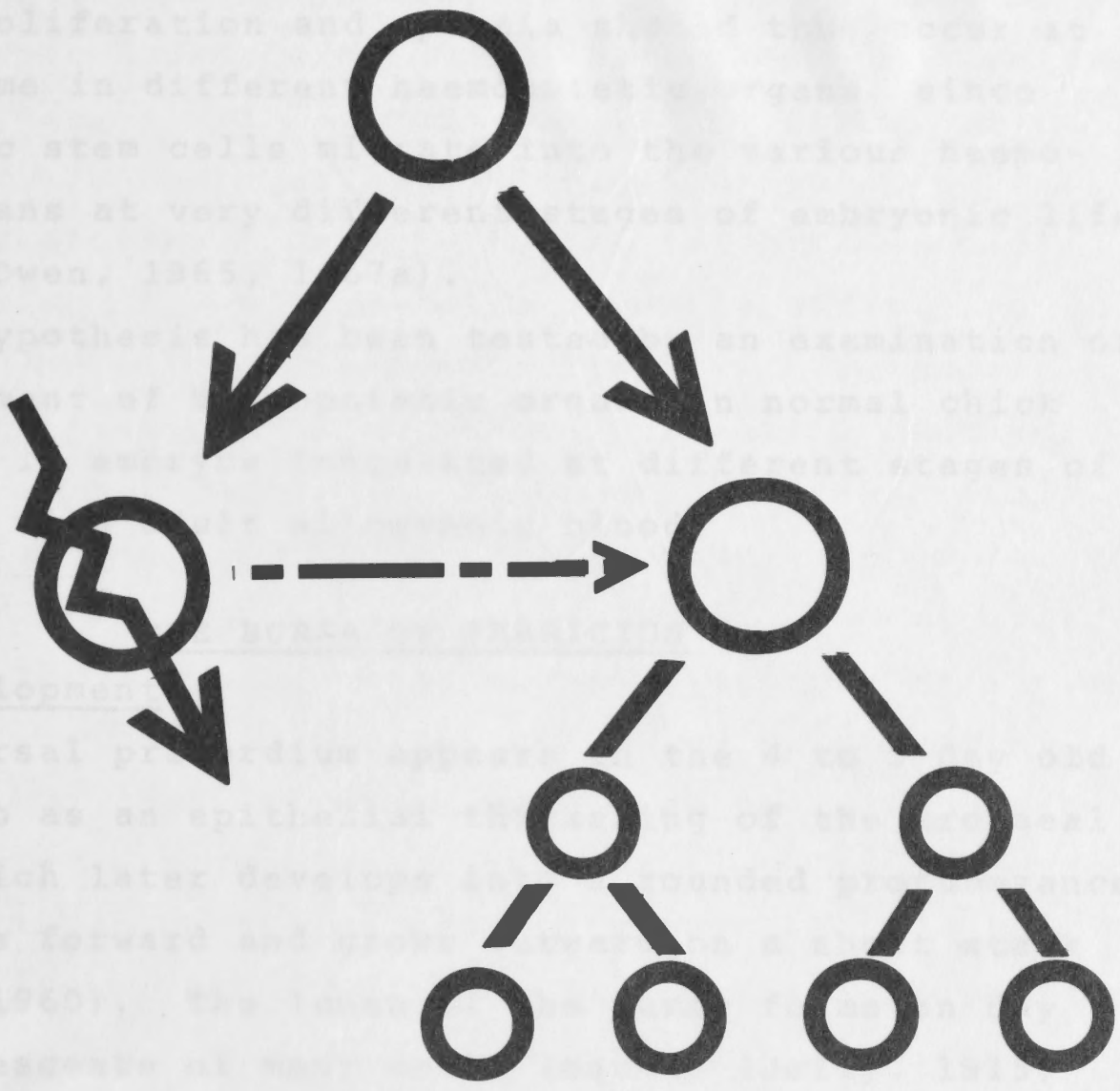
CHAPTER 7. CHANGES IN THE DEVELOPMENT OF EMBRYONIC HAEMOPOIETIC TISSUE DURING A GRAFT-VERSUS-HOST REACTION : BURSA, THYMUS AND BONE MARROW

Introduction

In chapter 3, it was suggested that proliferative changes, such as spleen enlargement and pock formation, during a GVHR in the chick embryo, are the result of an interaction between donor lymphocytes and haemopoietic stem cells of the host. However, in GVHRs of very young chick embryos, primitive haemopoietic stem cells in the blood islands of the yolk sac appear to be destroyed or inactivated by the presence of adult allogeneic lymphoid cells (chapter 6). Thus, if the original thesis of an interaction between donor lymphocytes and host haemopoietic stem cells is correct, somewhat more specialised, rather than primitive, haemopoietic stem cells must be involved.

It is suggested that the pathogenesis of a GVHR in the chick embryo depends on a dual interaction of allogeneic donor lymphocytes with elements of the embryonic haemopoietic system as shown in diagram 7.1. The effect on primitive haemopoietic stem cells present in the yolk sac, is inactivation or destruction, while more specialised yolk sac derived stem cells which have migrated into the developing intra-embryonic haemopoietic tissues are stimulated to proliferate.

Haemopoietic stem cells first appear extra-embryonically in the blood islands of the area vasculosa and then in the yolk sac (Danchakoff, 1908, 1916c; Edmonds, 1966). They eventually colonise presumptive intra-embryonic haemopoietic tissues such as the thymus, bone marrow and bursa (Moore and Owen, 1965, 1966, 1967a, b) in a manner similar to germ cells (Auerbach, 1970). If the development of proliferative lesions during a GVHR depends on the presence of haemopoietic stem cells within these tissues, then the time when these lesions can first be induced in any particular haemopoietic organ should coincide with its colonization by stem cells from the yolk sac. Before that time, the effect of donor lymphocytes on that organ would be expected to be aplasia since destruction of haemopoietic stem cells in the yolk sac would prevent its



INACTIVATION

PRIMITIVE
HAEMOPOIETIC STEM
CELL IN THE YOLK SAC

PROLIFERATION

YOLK SAC DERIVED
HAEMOPOIETIC STEM
CELL IN THE
LYMPHORETICULAR
TISSUE

diagram 7.1

subsequent colonisation. These two pathological changes, cellular proliferation and aplasia should thus occur at the same time in different haemopoietic organs, since haemopoietic stem cells migrate into the various haemopoietic organs at very different stages of embryonic life (Moore and Owen, 1965, 1967a).

This hypothesis has been tested by an examination of the development of haemopoietic organs in normal chick embryos and in embryos inoculated at different stages of development with adult allogeneic blood.

THE BURSA OF FABRICIUS

Normal development

The bursal primordium appears in the 4 to 5 day old chick embryo as an epithelial thickening of the urodaeal membrane which later develops into a rounded protuberance that rotates forward and grows outward on a short stalk (Romanoff, 1960). The lumen of the bursa forms on day 7 by the coalescence of many small lacunae (Jolly, 1915; Boyden, 1922). By 10 days, small projections appear on the inner surface of the bursal wall and these develop into longitudinal folds or plicae which gradually extend further into the lumen (Jolly, 1915).

Follicle formation commences by 12 to 13 days, when epithelial buds appear along the edges of the plicae. These buds are formed by proliferation within the surface epithelium and they remain separated from the underlying mesenchymal layer by the epithelial basement membrane (Jolly, 1915).

Developing lymphocytes are found within the epithelial buds by the 15th or 16th day. Shortly before hatching, an outer cortical layer is laid down, also containing developing lymphocytes and the epithelial bud eventually forms the medullary region of the mature follicle (Jolly, 1915; Ackerman and Knouff, 1959).

The origin of the lymphoid cells within the bursal follicle has been a matter of some dispute. Most early investigators including Schumacher, Retterer, Wenckebach, Stiedia and Lelièvre (Jolly, 1915) considered that the lymphocytes were directly derived from cells of the epi-

thelial bud. The observation of 'intermediate' forms between epithelial cells and lymphoblasts, both in the light microscope (Ackerman and Knouff, 1959) and in the electron microscope (Ackerman, 1962) led to a revival of this view.

However, as early as 1915, Jolly had suggested that lymphoid transformation of the epithelial bud depends on the immigration of lymphoid cells from the underlying mesenchyme. He observed accumulations of large amoeboid cells beneath the epithelium before epithelial budding began at 12 days incubation. These cells were strongly basophilic and had a round or oval nucleus with one or more nucleoli and a dense chromatin network. They were apparently penetrating the basement membrane and became insinuated between the epithelial cells. Similar morphological observations, however, have also been interpreted as evidence for a migration of epithelial cells into the mesenchyme (Ackerman and Knouff, 1959, 1964).

While morphological evidence alone cannot indicate with certainty the direction of migration, chromosome marker experiments (Moore and Owen, 1965, 1966) have demonstrated that lymphoid cells of the bursal follicle are derived from haemopoietic stem cells which migrate into the mesenchymal layer of the bursa.

Experimental results

Early stages in the development of bursal follicles

Early stages in the development of lymphoid follicles in the bursa were examined by light microscopy. Up to 10 days, the epithelial layer is composed of columnar epithelial cells and no cells infiltrating from the underlying connective tissue layer were seen (fig. 7.1A). By the 11th day (fig. 7.1B) the epithelial layer has decreased in height and is now composed of 2 or 3 layers of closely packed cells. A few darkly staining cells closely apposed to the epithelial layer are occasionally seen. In the 12 day bursa (fig. 7.1C), these cells occur more frequently. They are similar in appearance to the amoeboid cells described by Jolly (1915). At intervals along the epithelium of the 12 day bursa, small clusters of these darkly staining cells could be seen below and within the epithelial

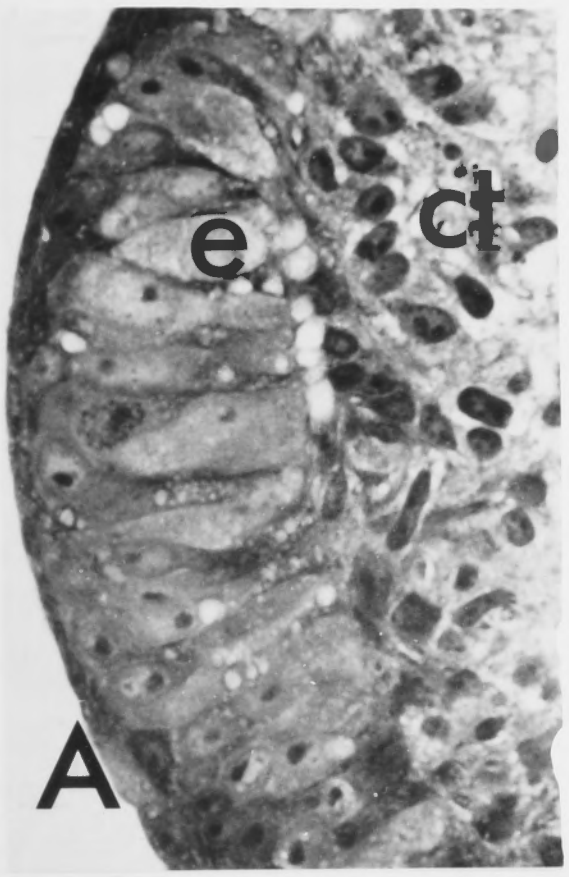
Fig. 7.1A-F Transverse sections of the bursal epithelium (e) in chick embryos aged 10 to 14 days.
Azure II/methylene blue Magnifications 900 x.

Fig. 7.1A 10 day bursa. At this stage the epithelium is composed of tall columnar cells separated from the connective tissue (ct) by a basement membrane.

Fig. 7.1B 11 day bursa. The epithelial layer has become flattened and in some areas darkly staining cells resembling haemopoietic stem cells (s) are closely applied to or apparently penetrating the epithelium.

Figs. 7.1C, D and E 12 day bursa. Three examples of haemopoietic stem cells (s) below the epithelium (fig. 7.1C), apparently penetrating it (fig. 7.1D) and within the epithelial layer (fig. 7.1E).

Fig. 7.1F 14 day bursa. An epithelial bud has formed in association with the invading cells.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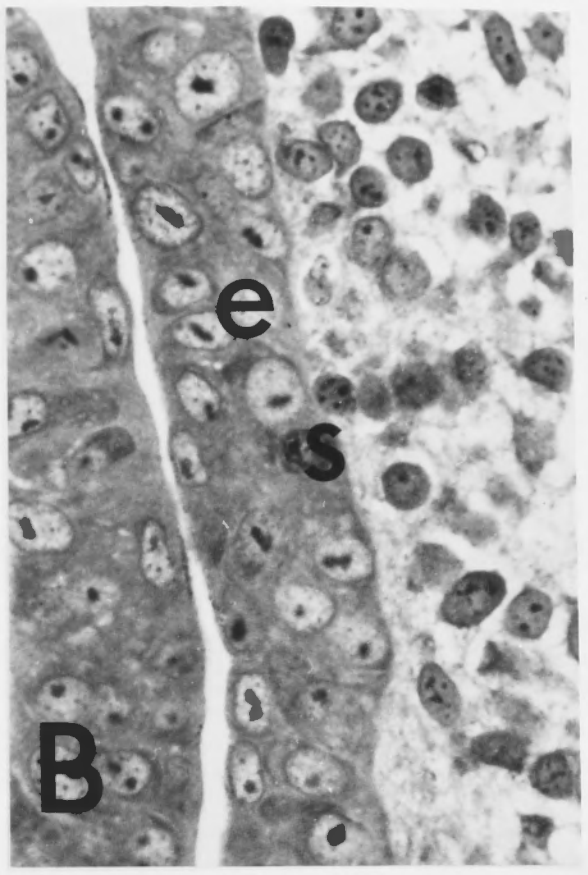
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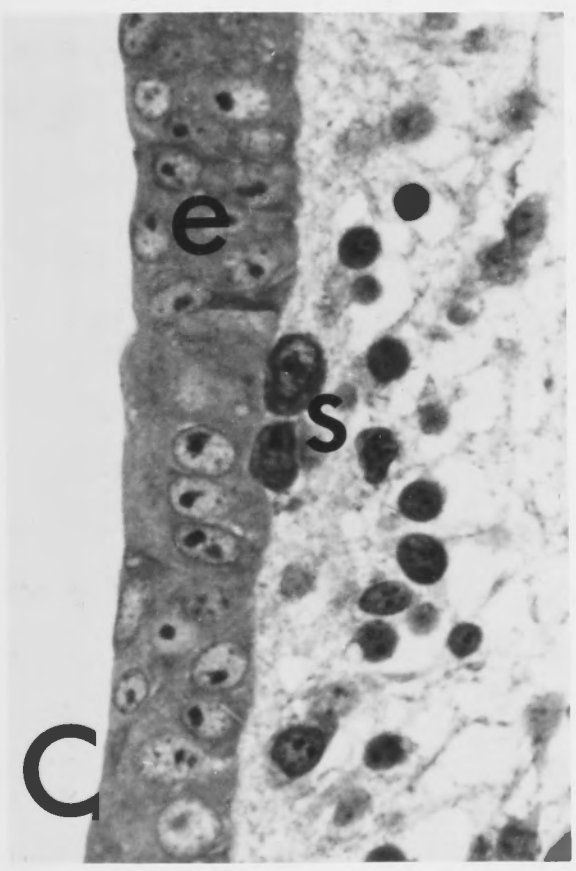
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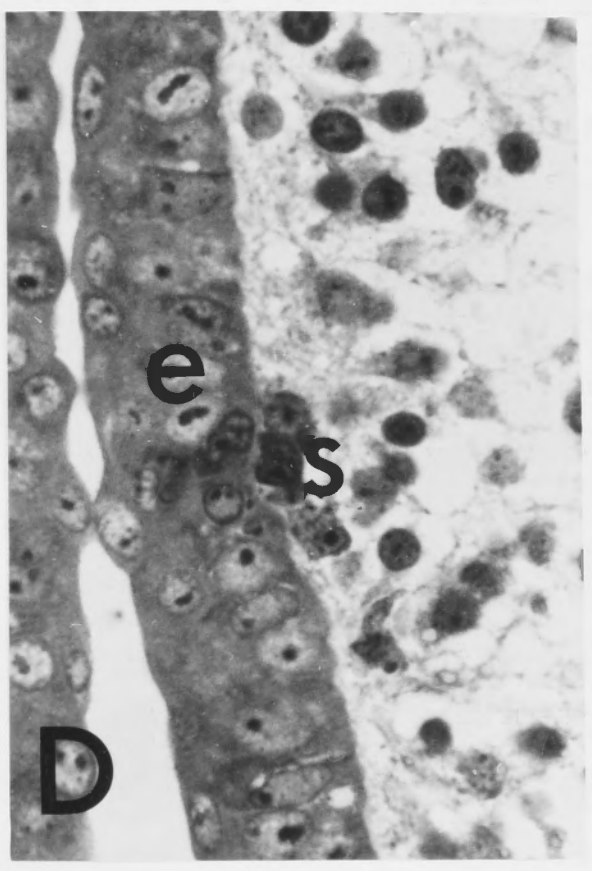
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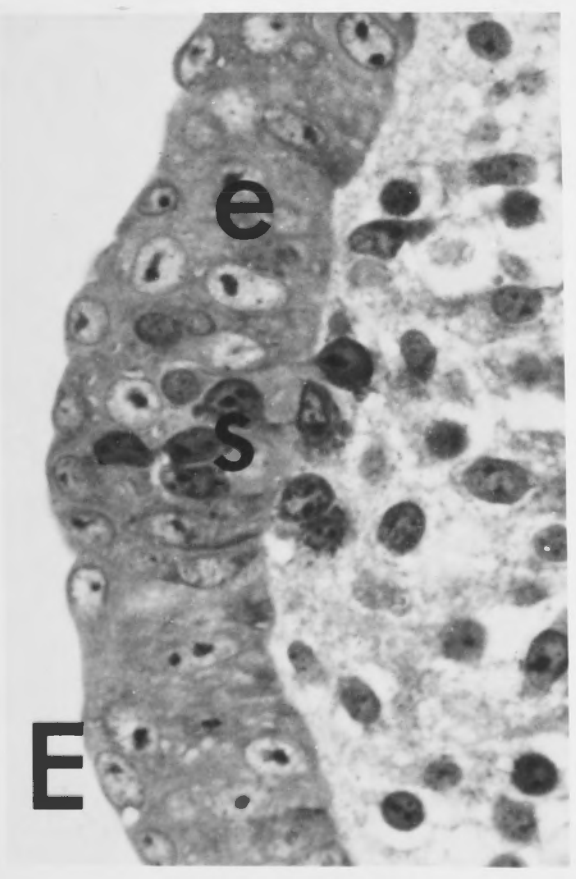
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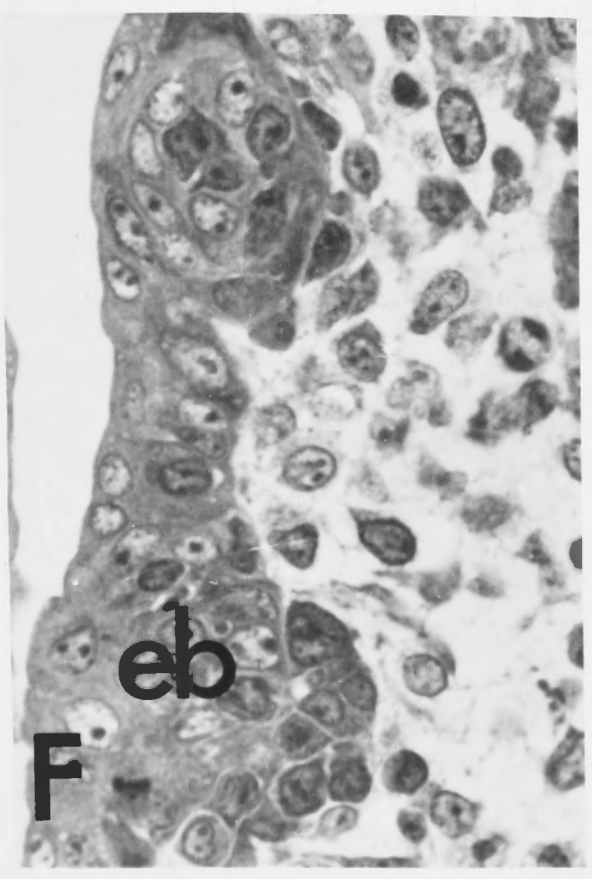
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layer (figs. 7.1D, E). The presence of darkly staining cells within the epithelium always coincided with clusters of similar cells in the underlying mesenchyme. By day 14, distinct follicles have formed (fig. 7.1F). Proliferation of epithelial cells, apparently around the invading cells, has resulted in an epithelial bud.

It was concluded that these infiltrating cells were haemopoietic stem cells which are known to invade the bursal epithelium at this stage of development (Moore and Owen, 1966).

The effect of a GVHR on the development of the bursa

The effect of a GVHR on the development of the bursa was examined by light microscopy in 5 to 14 day old randomly bred chick embryos, 6 days after the inoculation of 0.1 ml of a 1:1 dilution of adult AA blood in Alsever's solution on the CAM. Six to 10 embryos were examined in each age group.

5 day and 6 day recipients

In embryos inoculated with allogeneic blood at 5 or 6 days of age, the number of haemopoietic stem cells associated with or infiltrating the bursal epithelium 6 days later was greatly reduced. Very few stem cells were seen, either within or directly beneath the bursal epithelium of 5 day (fig. 7.2B) or 6 day (fig. 7.2D) recipients. Infiltrating stem cells, however, are readily found in the bursa of control embryos aged 11 (fig. 7.2A) or 12 (fig. 7.2C) days.

8 day and 10 day recipients

Initiation of a GVHR on, or after day 8, was found to depress the number of follicles which subsequently formed in the bursa. In the 8 day recipients (fig. 7.2F) only a few isolated epithelial buds were observed, while in comparable control animals, aged 14 days, numerous bursal follicles had developed (fig. 7.2E). The bursa of 16 day old embryos inoculated at day 10 (fig. 7.3B) had lower folds or plicae than the normal 16 day bursa (fig. 7.3A). The number of follicles formed in 10 day recipients was again depressed although follicles which had developed were generally larger than those observed in 8 day recip-

Fig. 7.2A-F Transverse sections from the bursa of normal 11 day to 14 day old randomly bred chick embryos compared with similar sections from the bursas of experimental animals inoculated 6 days previously with diluted adult AA blood. This series illustrates the effect of a GVHR on the migration of stem cells into the bursal epithelium and the subsequent depression of follicle development.
Azure II/methylene blue Magnifications 225 x.

Fig. 7.2A and fig. 7.2C Sections from a normal 11 day (fig. 7.2A) and 12 day (fig. 7.2C) bursa. Clusters of stem cells (indicated by arrows) can be seen, either closely applied to, or apparently infiltrating the bursal epithelium.

Fig. 7.2B and fig. 7.2D Stem cells can not be seen near the bursal epithelium of 11 day (fig. 7.2B) or 12 day (fig. 7.2D) chick embryos which had been inoculated with adult allogeneic blood. The appearance of the bursal epithelium and the underlying mesenchyme is, however, similar to that of control embryos of the same age.

Fig. 7.2E A normal 14 day bursa. Epithelial buds in association with infiltrating stem cells form clearly defined bursal follicles (f).

Fig. 7.2F Bursa from a 14 day embryo inoculated at 8 days with adult allogeneic blood. The bursa resembles that of a younger embryo since no follicles have developed.


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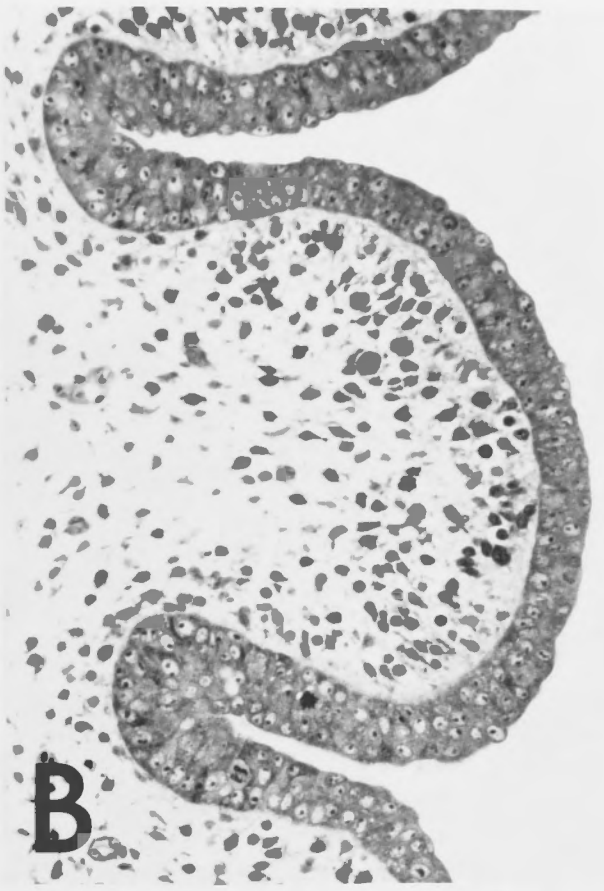
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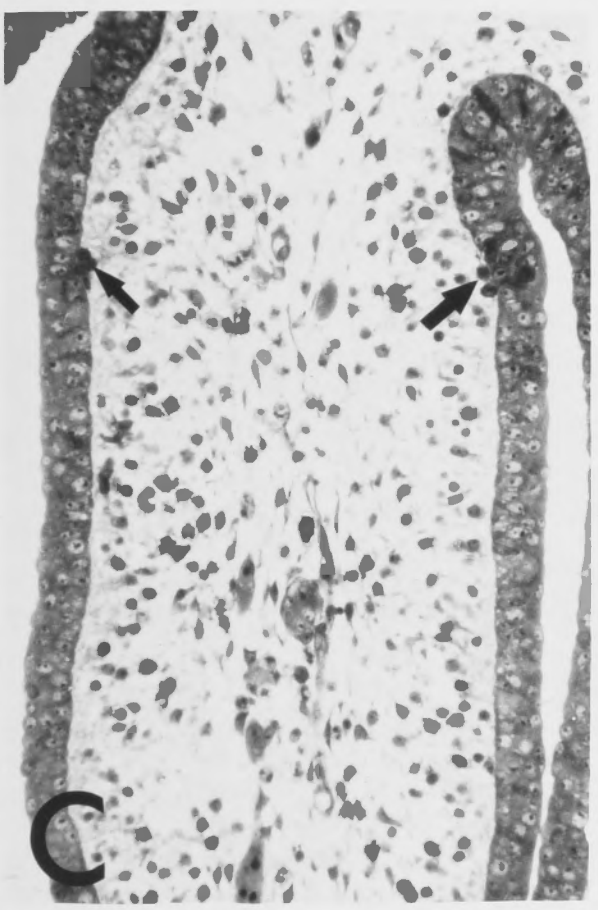
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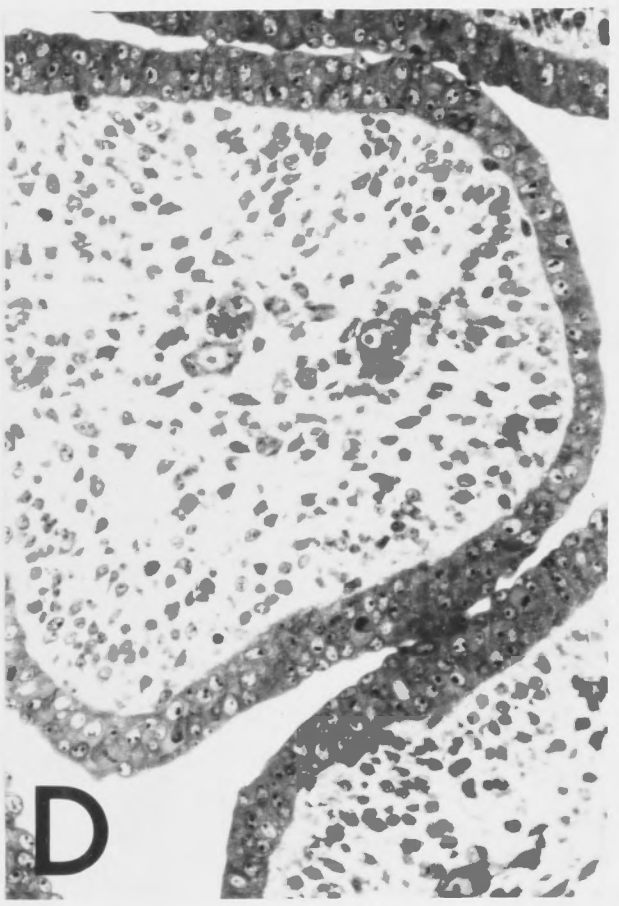
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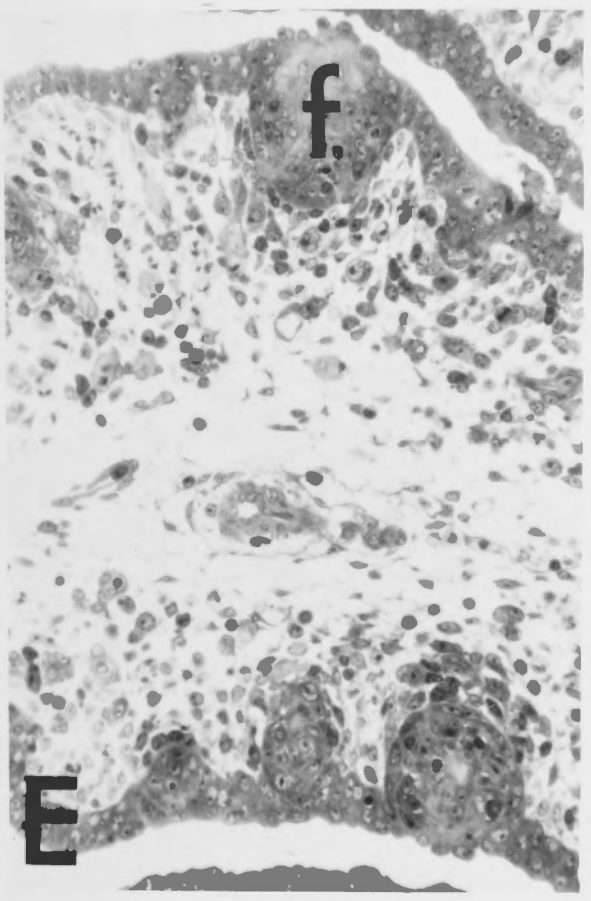
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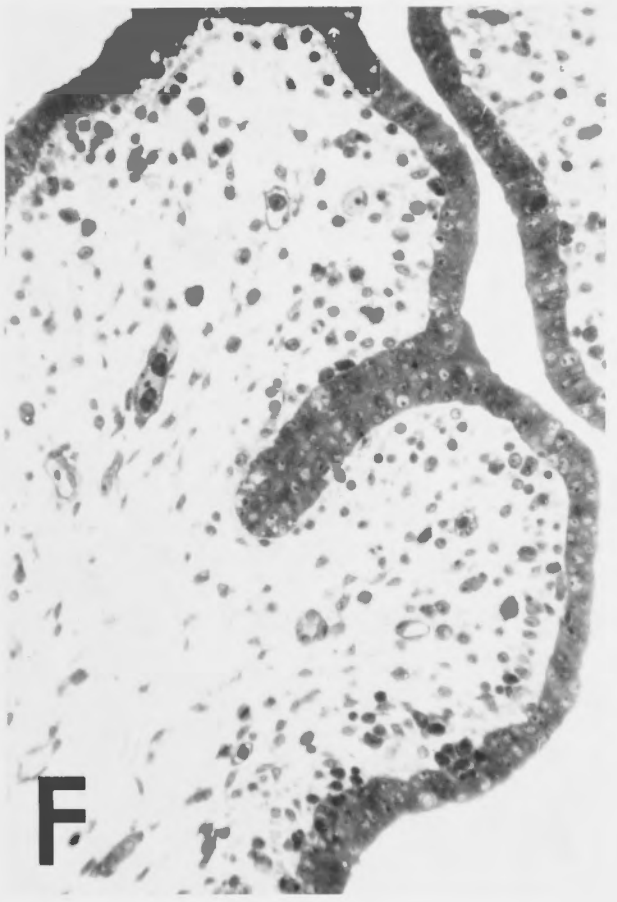
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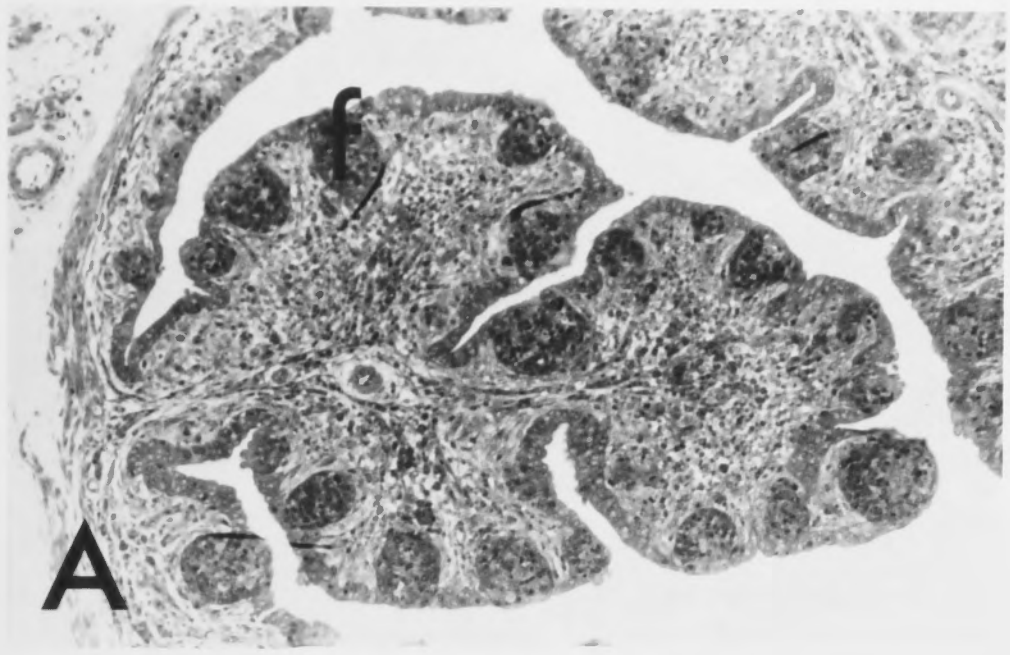
Fig. 7.3A-D Transverse sections from the bursa of normal 16 day and 20 day old randomly bred chick embryos compared with similar sections from embryos of the same age, 6 days after the inoculation of adult allogeneic blood.
Azure II/methylene blue Magnifications 100 x.

Fig. 7.3A and fig. 7.3C Normal 16 day bursa (fig. 7.3A) and 20 day bursa (fig. 7.3C) in which lymphoid follicles (f) are developing. By 20 days the follicles have become quite large and occupy most of the mesenchymal layer of the bursal fold.

Fig. 7.3B Bursa from a 16 day old embryo inoculated at 10 days with adult allogeneic blood. Only a few isolated follicles (f) have formed and these are smaller than those which are seen in control embryos of the same age (fig. 7.3A).

Fig. 7.3D Bursa from a 20 day old embryo inoculated at 14 days with adult allogeneic blood. The lymphoid follicles (f) are well developed and comparable in size to those seen in a control embryo (fig. 7.3C). A large focal accumulation of proliferating cells (p) has now developed within the mesenchymal layer.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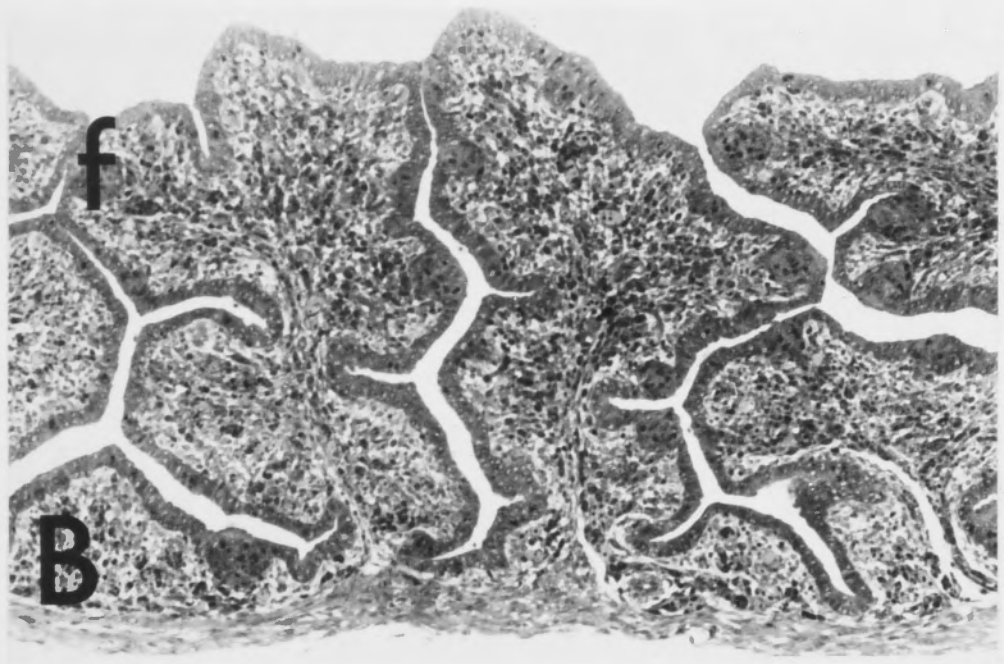
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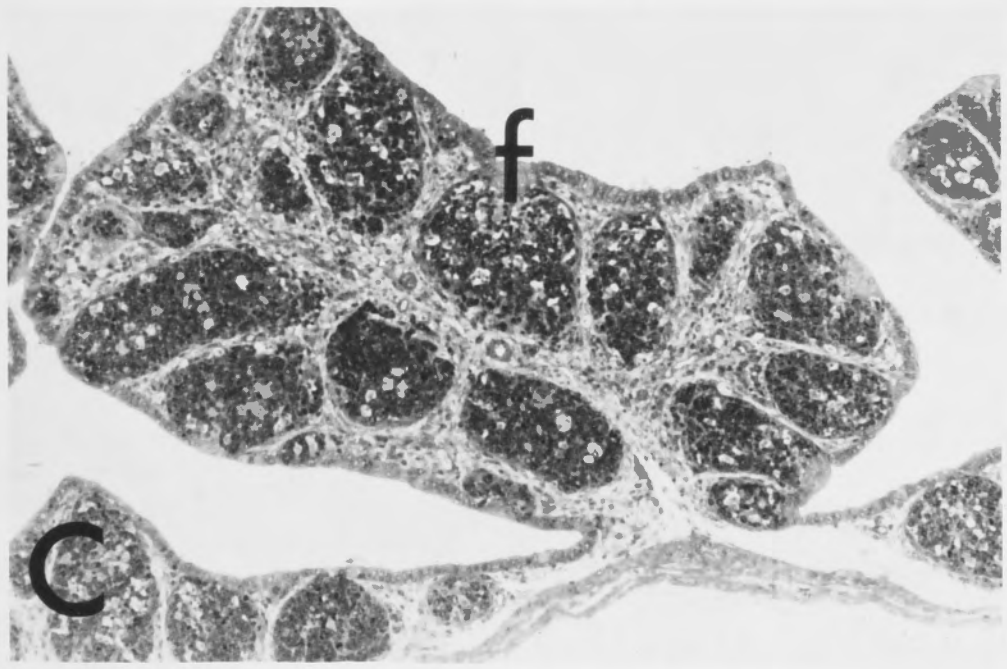
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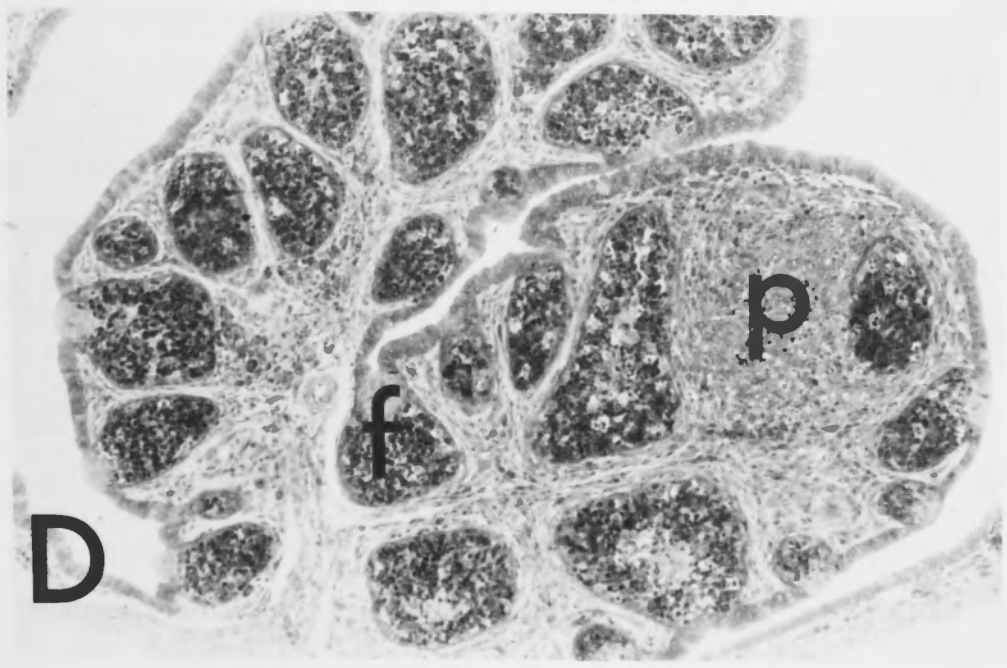
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ients but smaller than in the 16 day controls.

14 day recipients

Proliferative lesions were found within the bursa of all 14 day recipients (8/8) examined at day 20. They consisted of focal accumulations of 'reticular' cells within the mesenchymal layer of the bursal fold (fig. 7.3D). The follicles in the bursa of these embryos were often rather small although in some cases follicles of normal size were observed. The bursal folds were generally rather low. In comparison, the folds in the normal 20 day bursa (fig. 7.3C) extend into the centre of the bursal cavity. Closely spaced follicles project at intervals from the surface epithelium of the fold and fill most of the underlying connective tissue layer.

The effect of the graft-versus-host reaction on the structure of the bursal follicles

The changes in the structure of the follicle itself have been examined in greater detail.

5 day and 6 day recipients

In the normal 11 day (fig. 7.2A and fig. 7.4A) or 12 day (fig. 7.2C and fig. 7.4C) bursa, stem cells accumulate beneath the bursal epithelium and penetrate into this layer as already described. In experimental animals of the same age, inoculated with adult allogeneic blood at day 5 or day 6, the migration of stem cells into the bursal epithelium is greatly depressed. Very few, if any, haemopoietic cells are associated with the epithelial layer in those inoculated on the 5th day (fig. 7.4B) although some are occasionally found in embryos inoculated at day 6 (fig. 7.4D).

8 day and 10 day recipients

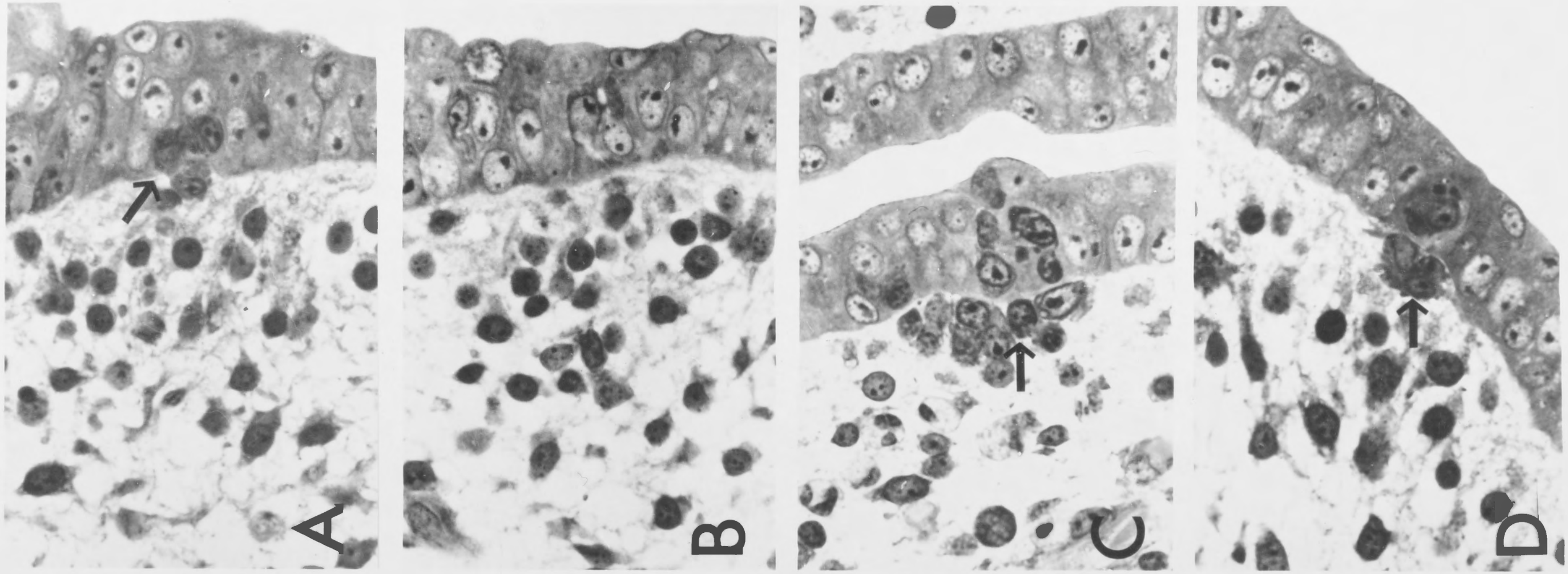
The normal 14 day bursa contains small but well developed follicles (fig. 7.5A) composed of darkly staining haemopoietic cells interspersed among lighter lympho-epithelial cells derived from the epithelial bud. In contrast, the follicles which form in the bursa of experimental animals inoculated at day 8 (fig. 7.5B) are much smaller and appear to be composed only of epithelial cells, although a few stem cells can be identified within the

Fig. 7.4A-D Transverse sections through the bursal epithelium of normal 11 and 12 day embryos and in experimental animals of the same age, inoculated at 5 or 6 days with adult allogeneic blood. These figures illustrate, at a higher magnification, the depression of stem cell migration into the bursal epithelium, during a GVHR.
Azure II/methylene blue Magnifications 1,000 x.

Fig. 7.4A and fig. 7.4C Normal 11 day (fig. 7.4A) or 12 day (fig. 7.4C) bursa in which stem cells (marked with an arrow) are seen apparently infiltrating the bursal epithelium or clustered directly beneath it.

Fig. 7.4B Bursa from an 11 day embryo inoculated at day 5 with adult allogeneic blood. Stem cells associated with the bursal epithelium were not seen in these recipients.

Fig. 7.4D Bursa from a 12 day embryo inoculated at day 6 with adult allogeneic blood. Although few stem cells were seen in these embryos, occasionally small clusters of cells were found beneath the epithelium as illustrated in this figure.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follicle or in the surrounding connective tissue.

The follicles in 16 day old embryos inoculated at day 10 (fig. 7.5D) are often of normal size but again appear to be composed almost entirely of epithelial cells with only a few darkly staining haemopoietic cells in or around the follicle. In the normal 16 day bursa (fig. 7.5C) the follicles contain many darkly staining cells which now have a heavily chromatic nucleus characteristic of developing lymphocytes (Venzke, 1952). These lymphoid cells are interspersed among large pale cells of apparent epithelial origin.

14 day recipients

A normal 20 day bursal follicle is illustrated in fig. 7.6. At this stage, the follicle is composed of a central medulla and an outer cortex separated by a basement membrane as described by Jolly (1915). Cells of the medulla, which derives from the epithelial bud and immigrant haemopoietic stem cells, consist of lymphocytes at various stages of development and a reticular network of modified, rounded epithelial cells. Epithelial cells of the medulla form a basal layer above the basement membrane and can also be identified in the epithelial tuft at the apex of the follicle.

The cortical layer derives from the surrounding mesenchyme in association with immigrant stem cells, and is an additional, although minor, site of lymphopoiesis. Numerous capillary vessels may penetrate the cortex but they do not enter the medulla (Jolly, 1915). Clusters of developing granulocytes occur in the connective tissue surrounding the follicle.

If this structure is compared with a follicle from the bursa of a 20 day old embryo inoculated at day 14 with adult allogeneic blood, a number of differences are apparent (fig. 7.7). The follicle is much smaller and consists only of the medullary region, which except for size, resembles that of the controls. Focal accumulations of proliferating cells are found in the connective tissue layer surrounding the follicle. At high magnification (fig. 7.8A) these cells resemble the primitive 'reticulum' cells described by Biggs and Payne (1961a, b).

Fig. 7.6 A photomontage of a follicle in the bursa of a 20 day old normal chick embryo. The inner medulla (m) contains lymphocytes in various stages of development (l) scattered among a reticulum of lympho-epithelial cells (r). A basal layer of epithelial cells (b) resting on a basement membrane (bm) separates the medulla from the outer, cortical region of the follicle (c). Some lymphopoiesis occurs in the cortex and developing granulocytes (g) are also seen. The follicle is capped by an apical epithelial tuft (et) comprised of cells resembling those in the bursal epithelium (e). The connective tissue layer (ct) surrounding the follicle contains many small blood vessels (bv).
Azure II/methylene blue Magnification 1,000 x.

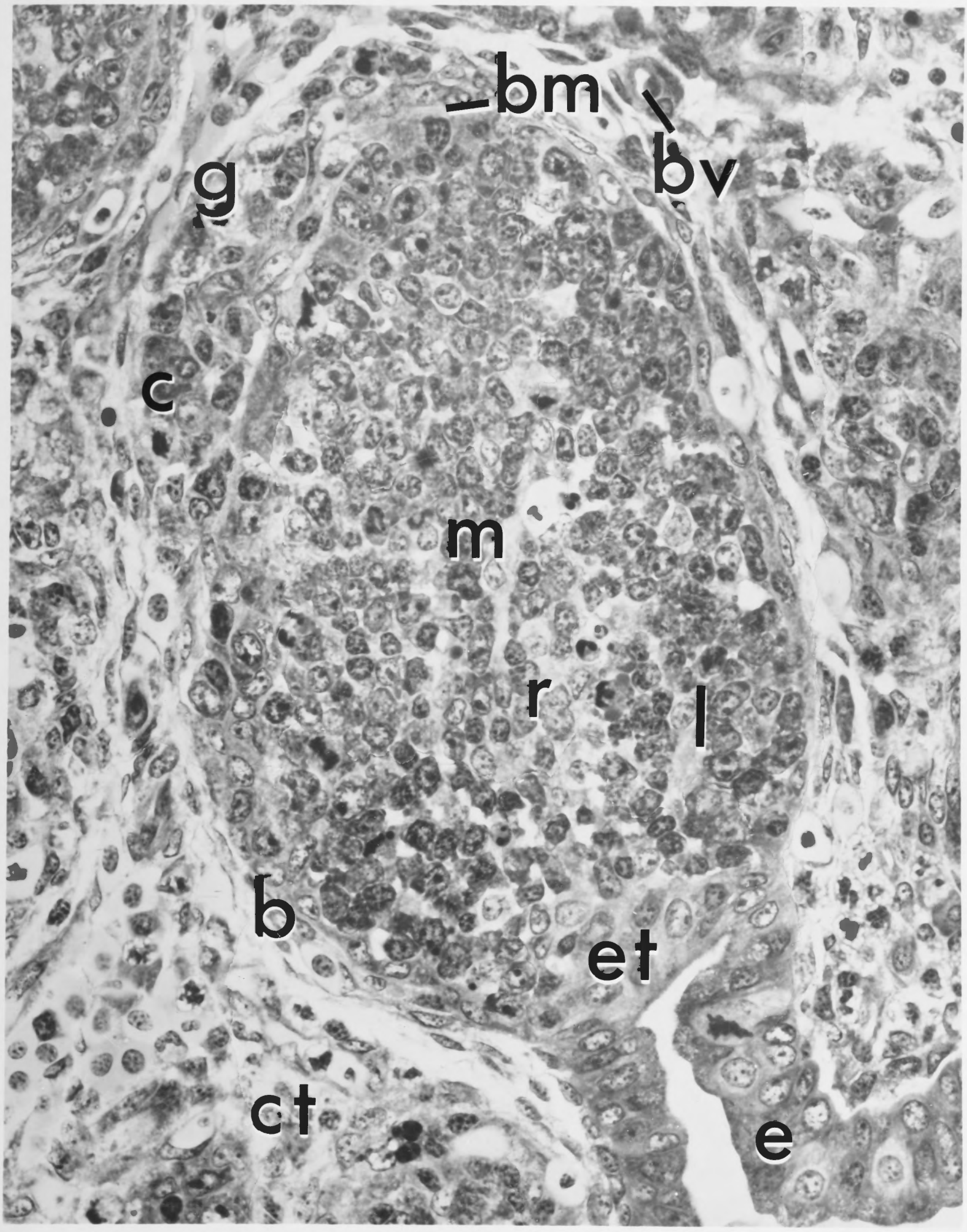
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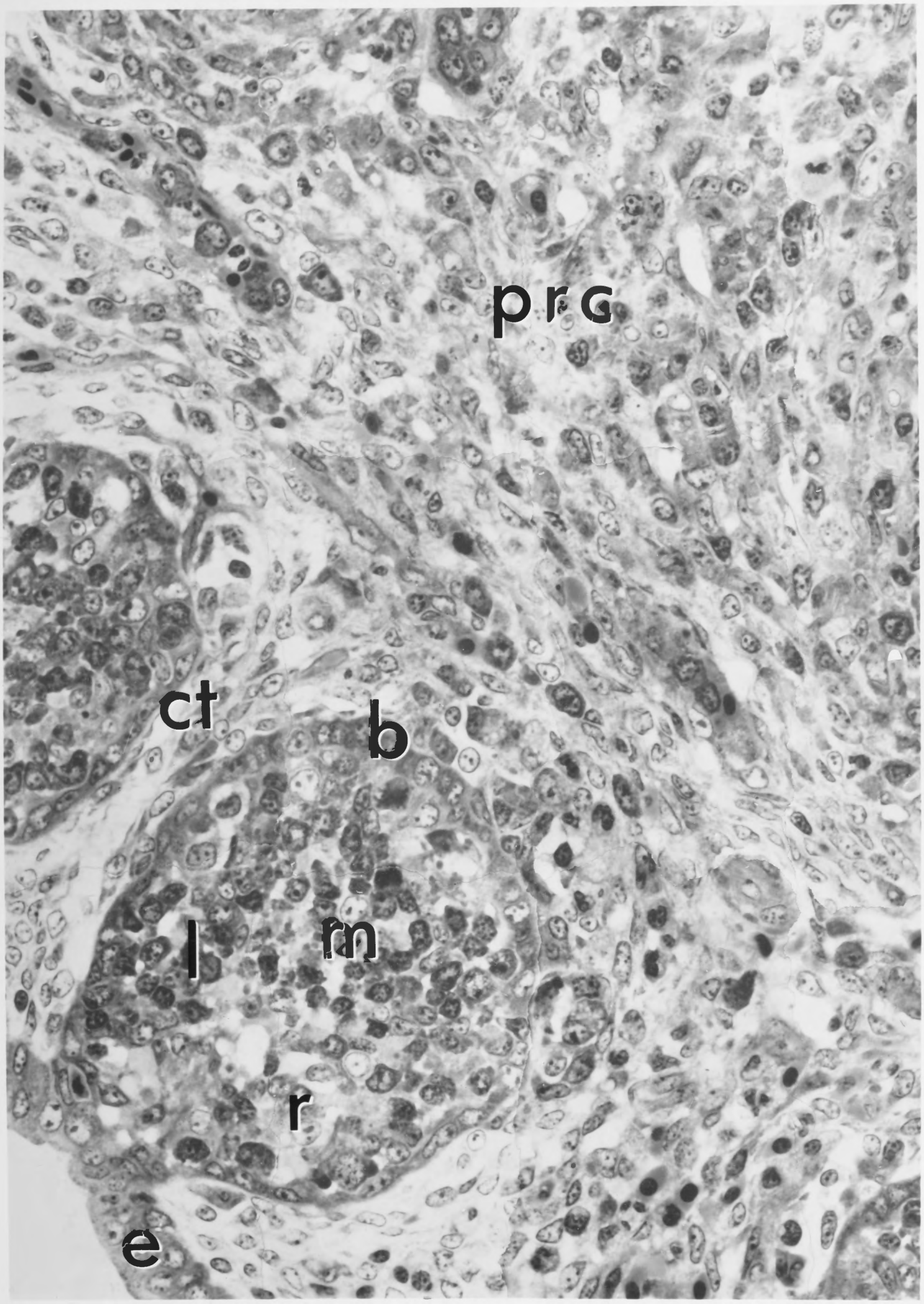
Fig. 7.7 A photomontage of a follicle in the bursa of a 20 day old chick embryo inoculated at 14 days with diluted adult allogeneic blood. The bursal follicle is much smaller than normal and a cortical layer has not differentiated. The medulla (m) contains developing lymphocytes (l) scattered among a reticulum of lympho-epithelial cells (r). In the absence of the cortex, the basal layer of epithelial cells (b) surrounding the medulla is clearly seen. A typical proliferative lesion, composed of primitive reticular cells (prc) has developed in the connective tissue layer (ct) surrounding the follicle. The bursal epithelium (e) appears normal.

Azure II/methylene blue Magnification 1,000 x.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The results of these experiments show that the age of the host determines the pathogenesis of lesions in the bursa during a GVHR. In very young embryos, inoculated at day 5 or day 6, migration of stem cells into the bursal epithelium is markedly depressed. In older embryos (inoculated at day 8 or day 10), the size and number of follicles is depressed and follicles which do develop show little lymphoid development. Proliferative lesions occur only in embryos inoculated on or after day 14.

The effect of the GVHR on granulopoiesis in the bursa

In the normal bursa, some developing granulocytic cells are usually seen within the mesenchymal layer of the bursal folds (Jolly, 1915). A large increase in the number of these granulocytic cells was observed in many of the chick embryos inoculated with adult allogeneic blood at day 6 or day 8. This change, however, was not seen in embryos inoculated at day 5 or on day 10 or day 14. Fig. 7.8B illustrates this infiltration of granulocytic cells in an embryo inoculated at 6 days of age. Almost the entire connective tissue layer of the bursal fold has been invaded by these cells. Fig. 7.8C shows a similar area at a higher magnification.

Within the 6 day age group, there appeared to be some correlation between the granulocytic response in the bursa and the incidence of proliferative lesions. Of 11 embryos inoculated with adult blood at 6 days, 8 had developed pocks on the CAM 6 days later. Of these animals, 5 showed a marked granulocytic infiltration of the bursa. In comparison, the remaining 3 embryos, which had not developed pocks on the CAM, did not appear to have an augmented number of granulocytic cells in the bursa.

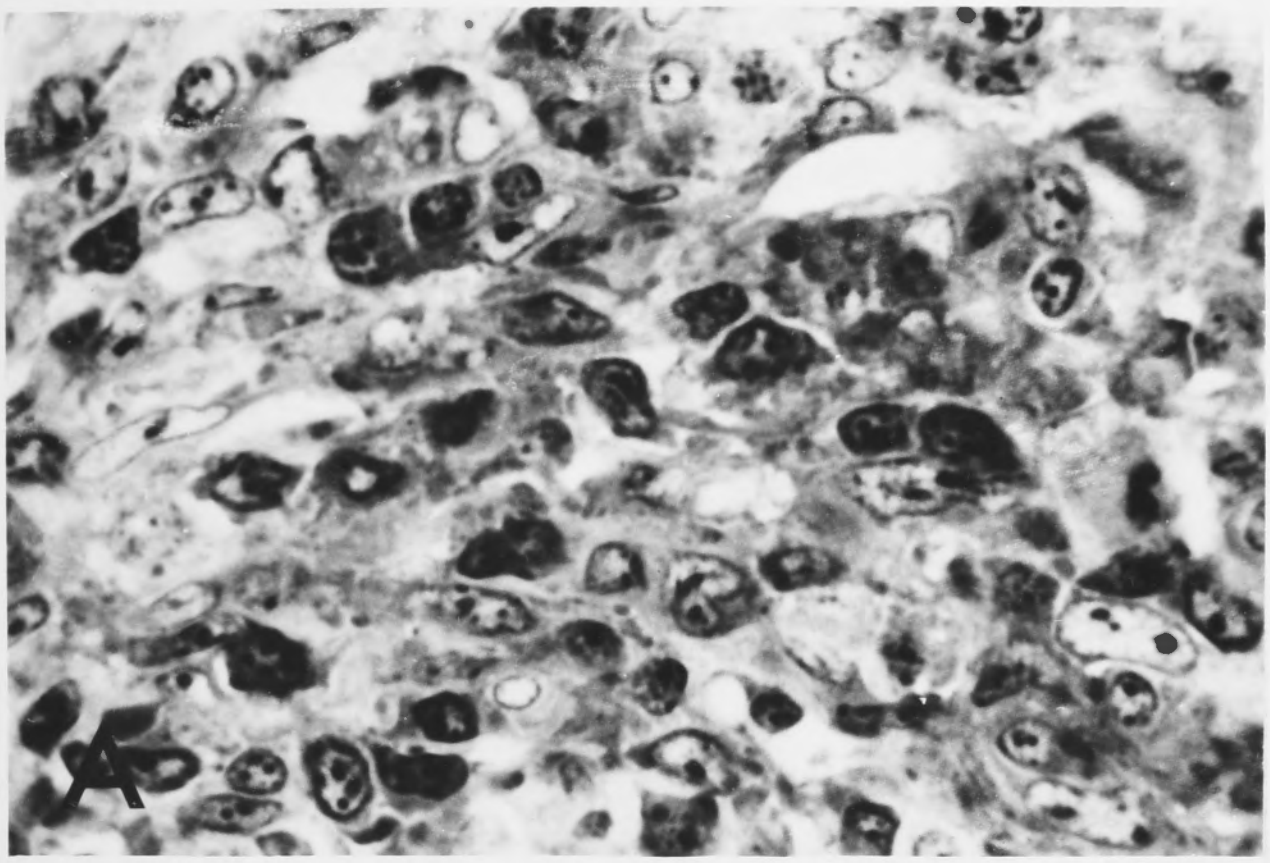
Specificity of changes in the bursa during a GVHR

To determine the specificity of the pathological changes which occur in the bursa during a GVHR further experiments were carried out with highly inbred recipients. Inbred CC or AA chick embryos of 6 to 14 days of age were inoculated on the CAM with 0.1 ml of a 1:1 dilution of adult AA blood in Alsever's solution and the bursa was examined 6 days later.

Fig. 7.8A Section through a focal lesion in the connective tissue layer of the bursa in a 20 day old embryo inoculated at 14 days with adult allogeneic blood. Proliferating cells in this lesion are primitive in appearance and resemble primitive reticular cells.
Azure II/methylene blue Magnification 1,300 x.

Fig. 7.8B Transverse section through the bursa of a 12 day embryo inoculated at 6 days with adult allogeneic blood, illustrating the accumulation of granulocytic cells (g) within the mesenchymal layer of the bursal fold.
Azure II/methylene blue Magnification 325 x.

Fig. 7.8C A detail at a higher magnification from the section shown in fig. 7.4B. The developing granulocytic cells (g) are more clearly seen, (b) marks a small blood vessel within the mesenchymal layer.
Azure II/methylene blue Magnification 1,300 x.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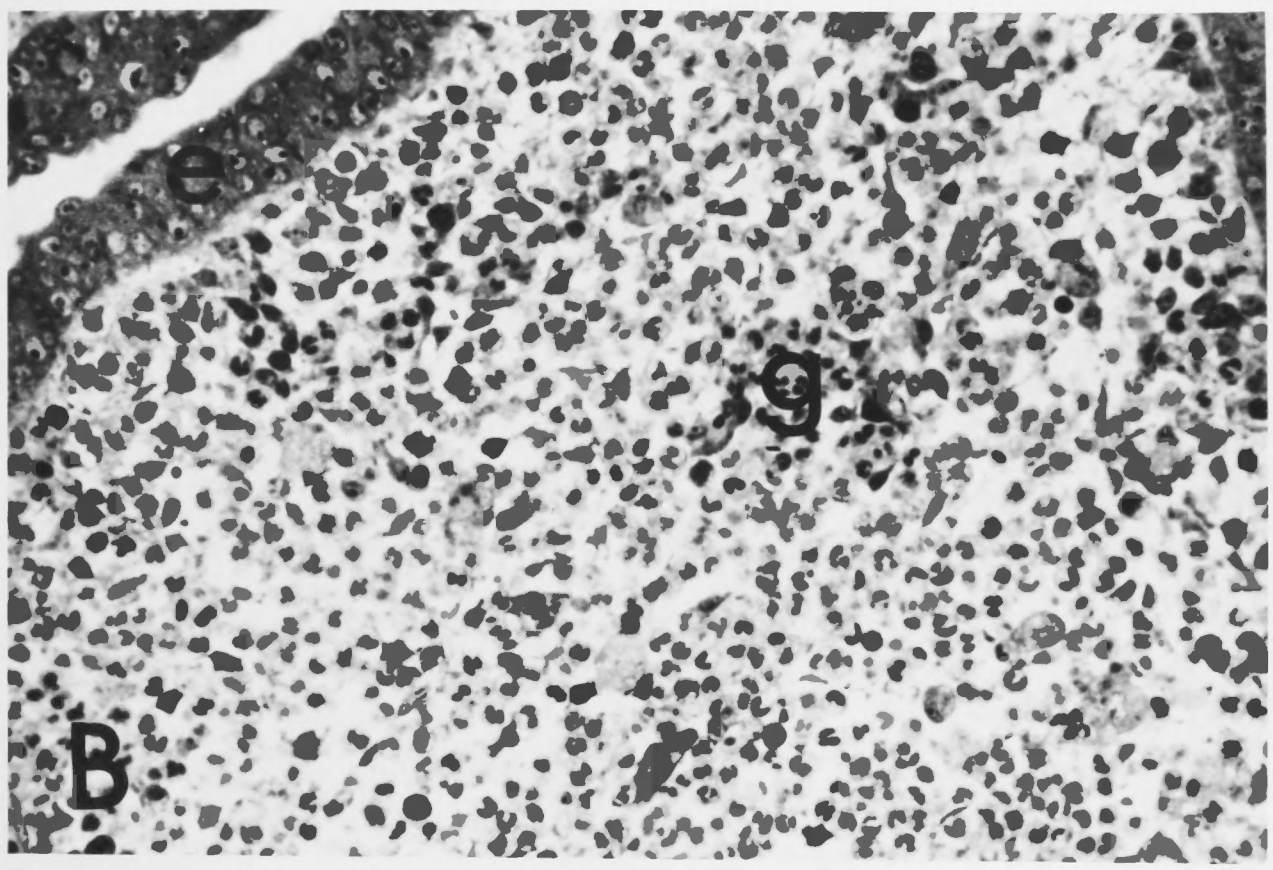
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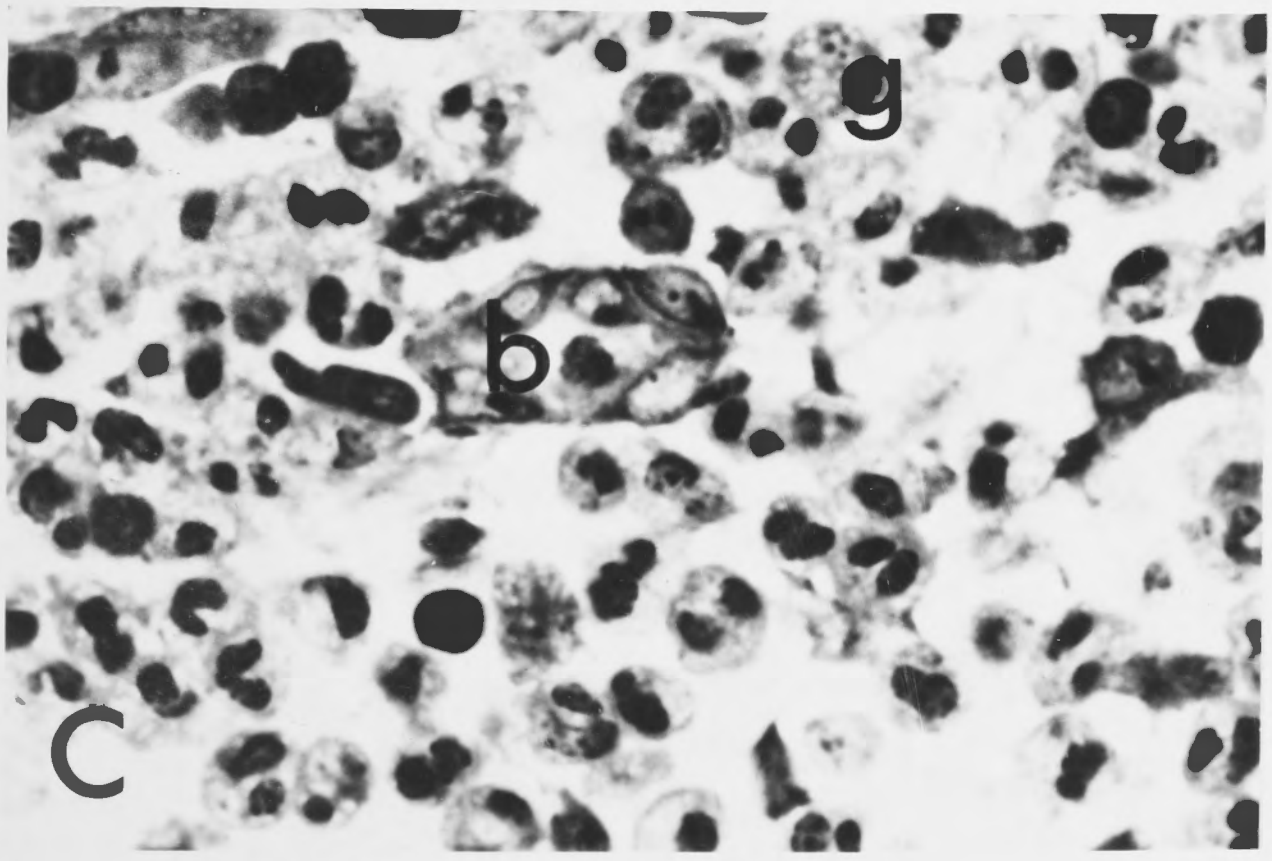
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No pathological changes could be detected in the bursa of AA embryos inoculated with syngeneic adult blood. The changes which occurred in CC embryos inoculated with allogeneic blood, however, were similar to those described in randomly bred recipients. Some of these inbred CC embryos inoculated at day 6 had haemorrhages on the body surface similar to those described in chapter 4. The bursa of these embryos was very poorly developed (fig. 7.9). The mesenchymal layer of the bursal folds was poorly differentiated, contained a few blood vessels and no granulocytic cells. There was also an absence of stem cells within, or directly beneath the epithelial layer.

The effect of inoculation at 6 days on subsequent follicle development was examined in a group of CC embryos. Four of the 24 embryos inoculated survived until day 18 when the bursa was examined. Fig. 7.10A illustrates the poor differentiation of the bursa in these recipients. Some follicles had developed but these were very small and consisted almost entirely of epithelial cells. Only a small number of darkly staining cells could be found, either within or around the epithelial buds (fig. 7.10B). The mesenchymal layer of the bursal fold contained many granulocytic cells (fig. 7.10A, fig. 7.10C). Despite the age of the embryos and the length of time after inoculation, no focal proliferative lesions similar to those seen in 14 day recipients were observed.

A quantitative estimation of the depression in follicle formation during a GVHR

An attempt was made to quantitate the degree to which follicle formation was depressed by a GVHR. Bursas from normal randomly bred embryos and from embryos inoculated with adult allogeneic blood at 5 to 14 days were sectioned across the equatorial plane which can be done accurately with transparent glycol methacrylate blocks. The number of follicles in each of these sections was counted and expressed as the ratio of number of follicles to the number of plicae or bursal folds. Comparison of these ratios in inoculated and control groups (fig. 7.11A) showed that follicle formation was significantly depressed ($p<0.01$) in 8 day, 10 day and 14 day recipients.

Fig. 7.9 Transverse section of the bursa from a 12 day old CC chick embryo inoculated at 6 days with diluted adult AA blood to show the poor development of the bursa in very young inbred hosts undergoing a GVHR. This may be compared with the normal 12 day bursa shown in fig. 7.2C.

Azure II/methylene blue Magnification 600 x.

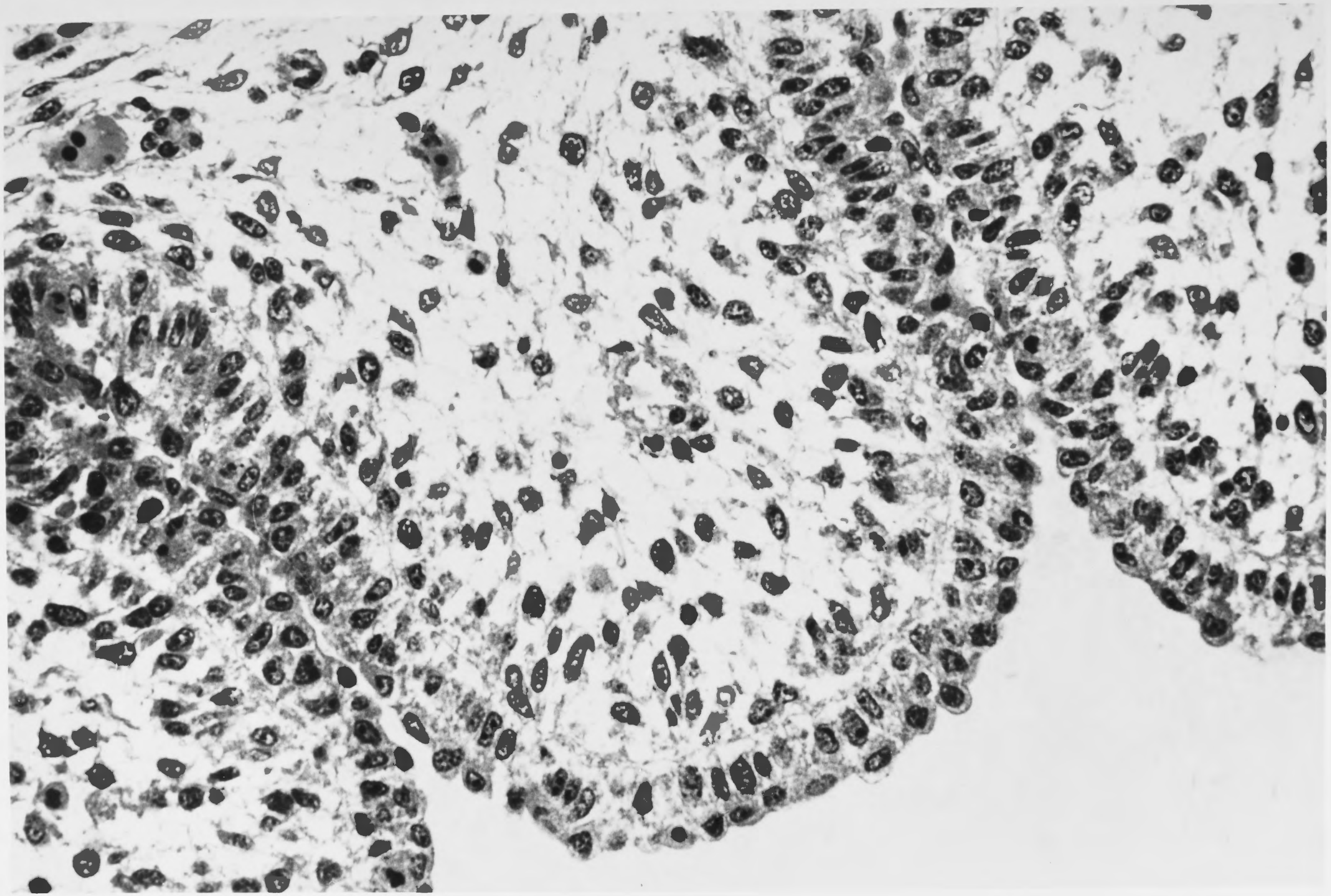
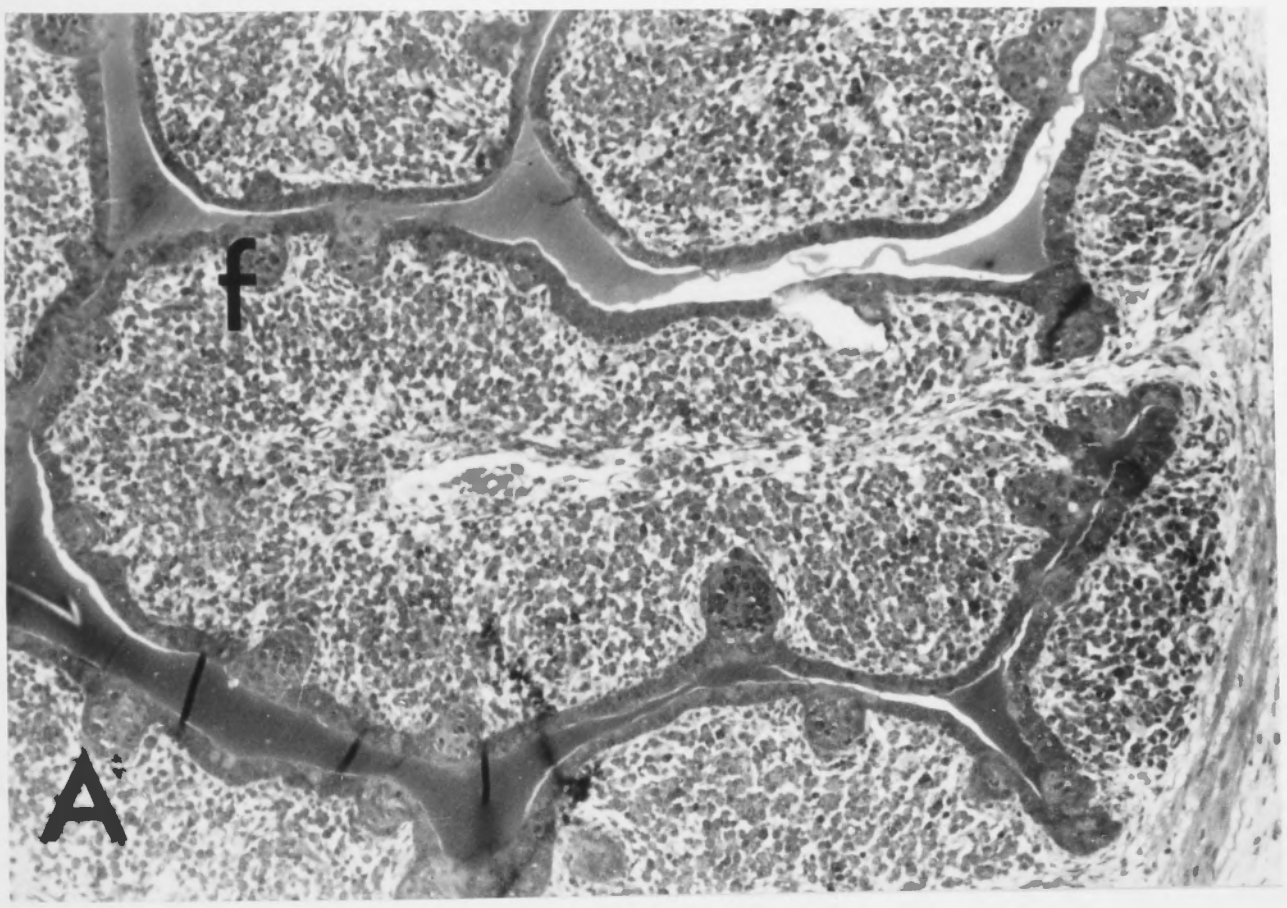


Fig. 7.10A Transverse section through the bursa of an 18 day old CC chick embryo inoculated at 6 days with adult allogeneic blood. Few lymphoid follicles have developed along the edges of the bursal epithelium, the underlying connective tissue contains many granulocytic cells but there are no focal proliferative lesions.
Azure II/methylene blue Magnification 140 x.

Fig. 7.10B Detail of a bursal follicle from the same section. Most of the cells in the follicle resemble cells of the epithelial tuft (et) or the adjacent epithelial layer (e). Darkly staining haemopoietic cells cannot be readily identified either within the follicle or in the surrounding connective tissue.
Azure II/methylene blue Magnification 1,400 x.

Fig. 7.10C Detail from the connective tissue layer which is filled with granulocytic cells (g).
Azure II/methylene blue Magnification 1,400 x.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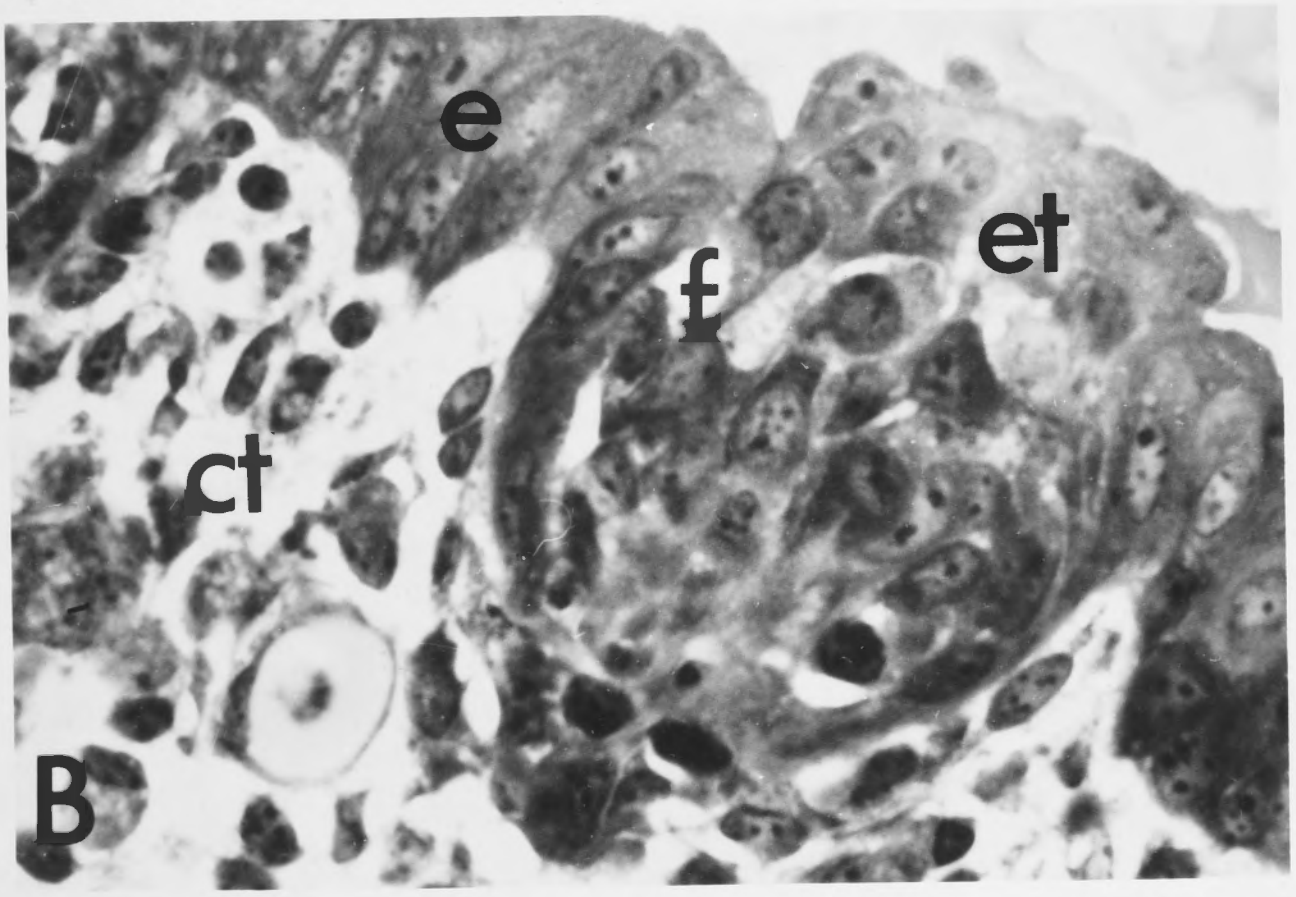
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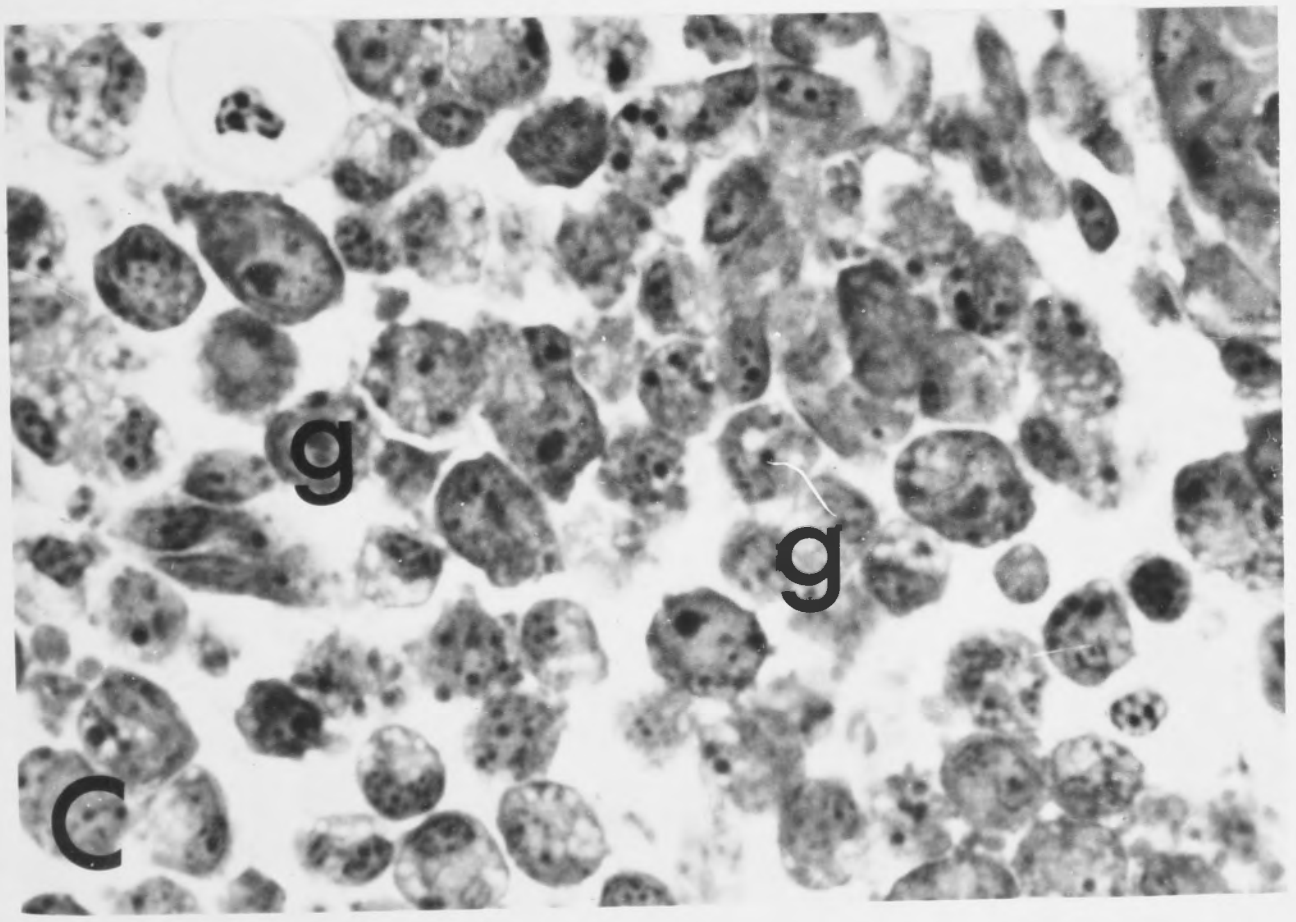
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Fig. 7.11A A comparison of the average number of follicles per pliça in the bursa of randomly bred control embryos and in the bursa from an experimental group of randomly bred embryos of the same age, examined 6 days after the inoculation of diluted adult AA blood on the CAM. Six to 12 embryos were examined in each group. The vertical lines represent the standard error.

Fig. 7.11B The development of a GVHR did not affect the average wet weight of the bursa. The closed circles in this figure indicate the average wet weight of the bursa in randomly bred chick embryos, 6 days after the inoculation of adult allogeneic blood. The average wet weight of the bursa in normal embryos of the same age is marked by open circles. Twelve or more embryos were examined in each group. The vertical lines represent the standard error.

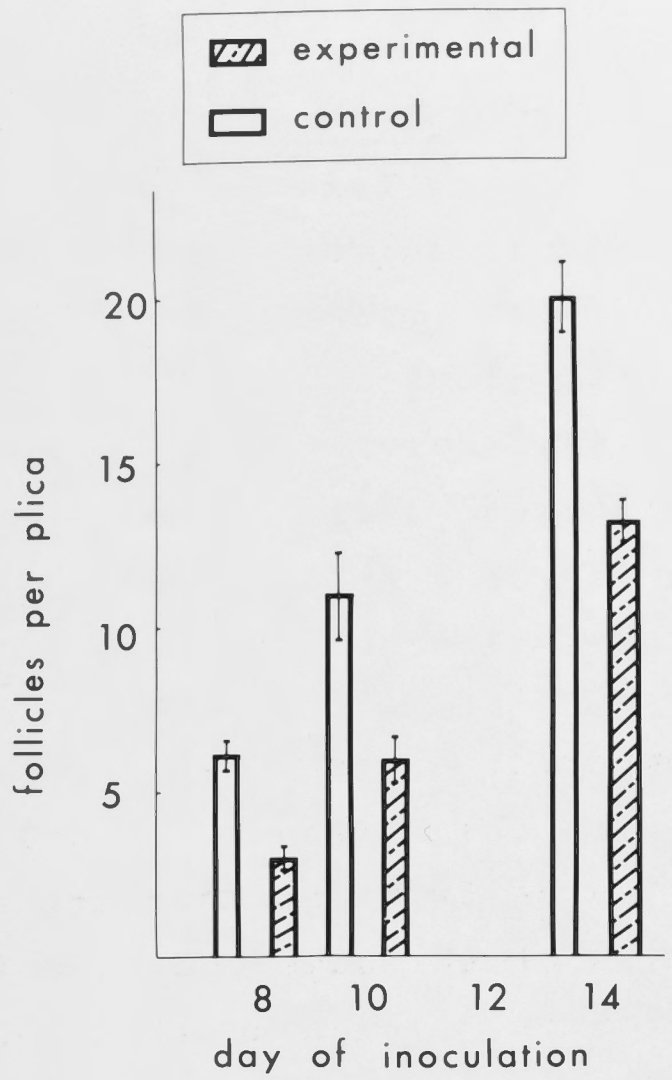


fig.7.11 A

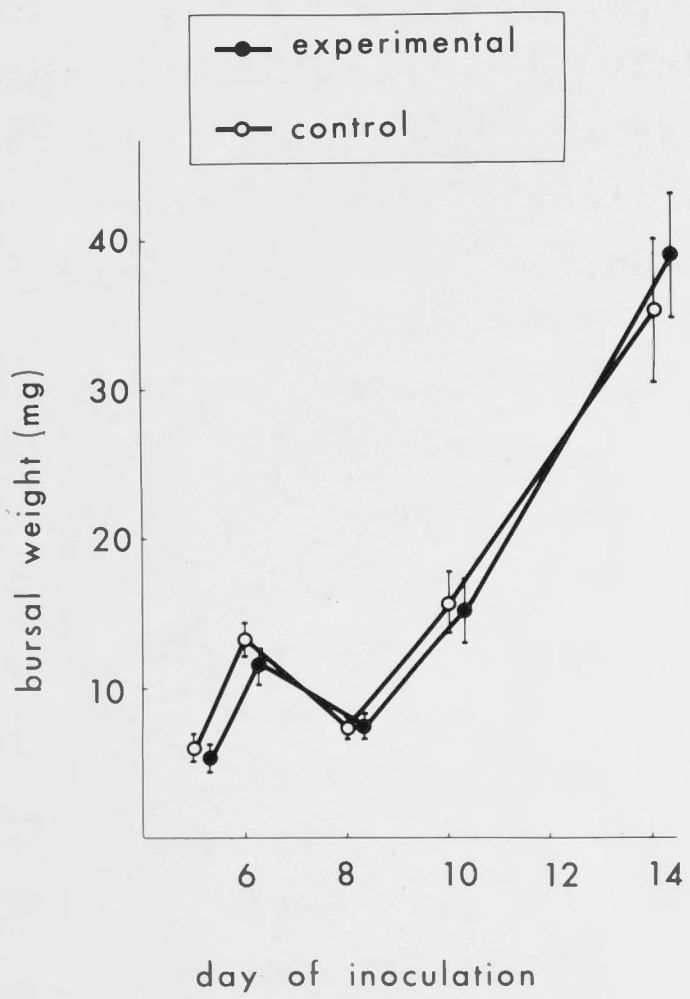


fig.7.11 B

The effect of a GVHR on the weight of the bursa

An experiment was carried out to determine whether the depression of follicle formation in the bursa was reflected by a decrease in bursal weight. The wet weight of this tissue was measured in normal embryos and in randomly bred embryos inoculated 6 days previously with adult allogeneic blood. As shown in fig. 7.11B, depression of follicle formation had little effect on the wet weight of the bursa. There was no significant difference between the wet weight of the bursa in experiment animals and the wet weight of the bursa in control embryos.

THE THYMUS

Normal development

In the chicken the thymus is derived from the epithelium of the 3rd and 4th pharyngeal pouches (Venzke, 1952; Schrier and Hamilton, 1952; Hammond, 1954) and first appears in the 7 day embryo as a mass of epithelial cells proliferating beside the jugular vein (Venzke, 1952). Lobule development and the differentiation of thymocytes begins around 9 days (Venzke, 1952).

The origin of thymic lymphocytes has been extensively debated. Some workers concluded that in the chick embryo (Danchakoff, 1908b; Venzke, 1952) as well as in other species (Norris, 1938; Klapper, 1946; Murray, 1947; Weiss, 1963; Smith, 1965; Hoshino *et al.*, 1969) thymocytes have a connective tissue origin. Others, however, maintained that thymic epithelial cells give rise directly to lymphocytes (Sainte-Marie and Leblond, 1958; Ackerman and Knouff, 1959, 1964, 1965; Auerbach, 1960, 1961; Knouff and Ackerman, 1963; Weakley *et al.*, 1964).

The latter theory has been largely refuted by the karyological studies of Moore and Owen (Solomon, 1971). Chromosome analysis of the thymus from parabiosed chick embryos (Moore and Owen, 1965, 1967) and of thymic grafts placed on the CAM (Moore and Owen, 1967; Owen and Ritter, 1969) demonstrated that the development of thymocytes depends on an inflow of blood-bourne stem cells which first enter the thymic anlage at 7 to 8 days incubation. These results agree with the histological findings of Venzke (1952) who described the accumulation of large lymphocytes

in the mesenchymal layer near the thymic primordium at day 7 (165 hours). At 7½ days (177 hours) a few large lymphocytes appeared to have migrated into the thymus and shortly afterwards the lymphocyte content of this tissue greatly increased. An apparent immigration of lymphoid cells has also been observed in the thymus of other species (Yadav and Papadimitriou, 1969; Hoshino et al., 1969).

Experimental results

The effect of a GVHR on the development of the thymus

The thymus of randomly bred chick embryos of different ages was examined by light microscopy 6 days after the inoculation of 0.1 ml of a 1:1 dilution of adult AA blood in Alsever's solution onto the CAM and compared with the thymus of normal embryos of the same age.

5 day recipients

In normal 11 day embryos the thymus has a lobular structure and blood vessels have penetrated the interlobular septae (fig. 7.12A). At high magnification (fig. 7.12C) many cells are seen with a heavily chromatic nucleus and a thin layer of cytoplasm which characterise developing small lymphocytes (Venzke, 1952). These cells are scattered among larger, more lightly staining cells with a sparsely chromatic nucleus which resemble the thymic epithelial cells described by Venzke (1952).

At 11 days the thymus of embryos inoculated at 5 days with adult allogeneic blood is still very small (fig. 7.12B). At higher magnifications (fig. 7.12D) only a few cells with a heavily chromatic nucleus can be seen, epithelial cells appear to predominate. This suggests a depression of lymphopoiesis in the thymus of these recipients. None of the 8 inoculated embryos examined at this stage had proliferative lesions in the thymus.

6 day recipients

The appearance of the thymus in normal 12 day embryos is similar to that seen at 11 days (fig. 7.13A, fig. 7.13C). In the experimental animals, small proliferative lesions were found in the thymus of 3 of the 8 embryos which had been inoculated at 6 days (fig. 7.13B). They consisted of clusters of pale staining cells which showed little differ-

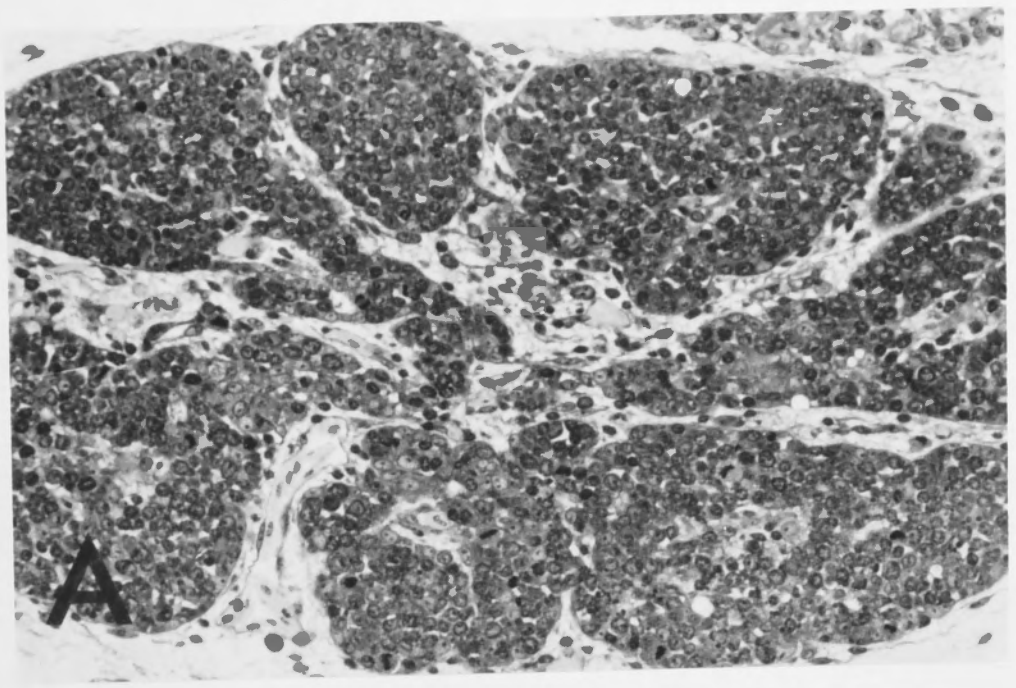
Fig. 7.12A-D Transverse sections from the thymus of an 11 day embryo inoculated at 5 days with adult allogeneic blood and from normal embryos of the same age. These sections illustrate the effect of the GVHR on lymphopoiesis in the thymus at this stage.

Fig. 7.12A Low power light micrograph of a normal thymic lobe (t) surrounded by connective tissue (ct).
Azure II/methylene blue Magnification 250 x.

Fig. 7.12B A similar section from the thymus of a 5 day recipient. The thymic lobe (t) appears smaller than normal. (ct) marks the surrounding connective tissue.
Azure II/methylene blue Magnification 250 x.

Fig. 7.12C The thymus of a normal 11 day embryo at a higher magnification to show the developing lymphocytes (l) and thymic epithelial cells (e).
Azure II/methylene blue Magnification 1,000 x.

Fig. 7.12D Most of the cells in the thymus of a 5 day recipient of the same age resemble thymic epithelial cells (e). Few developing lymphocytes (l) can be identified.
Azure II/methylene blue Magnification 1,000 x.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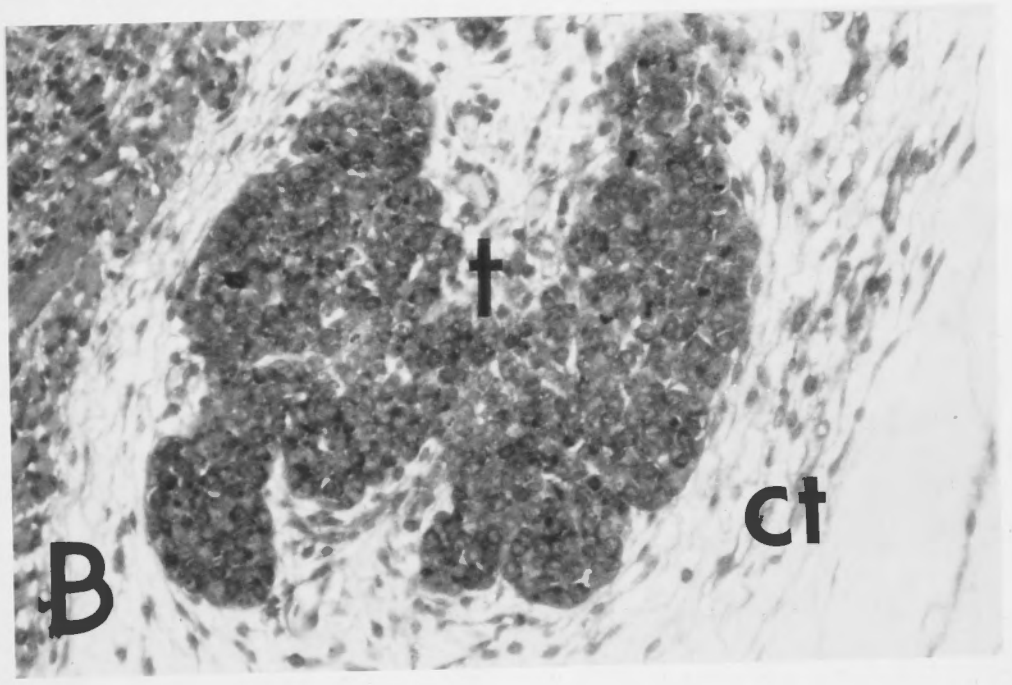
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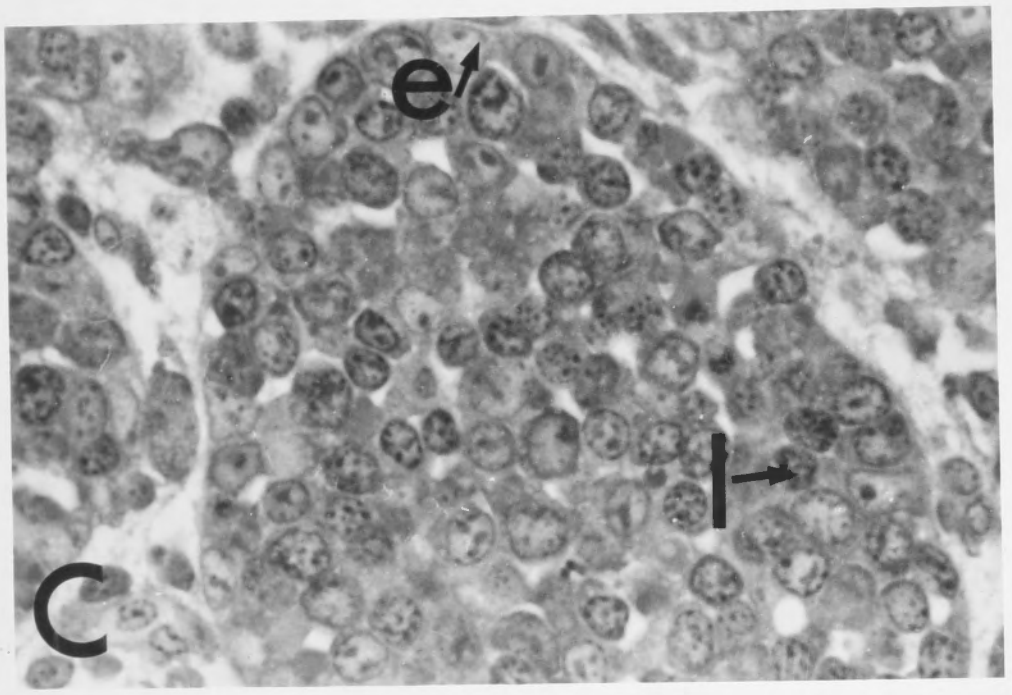
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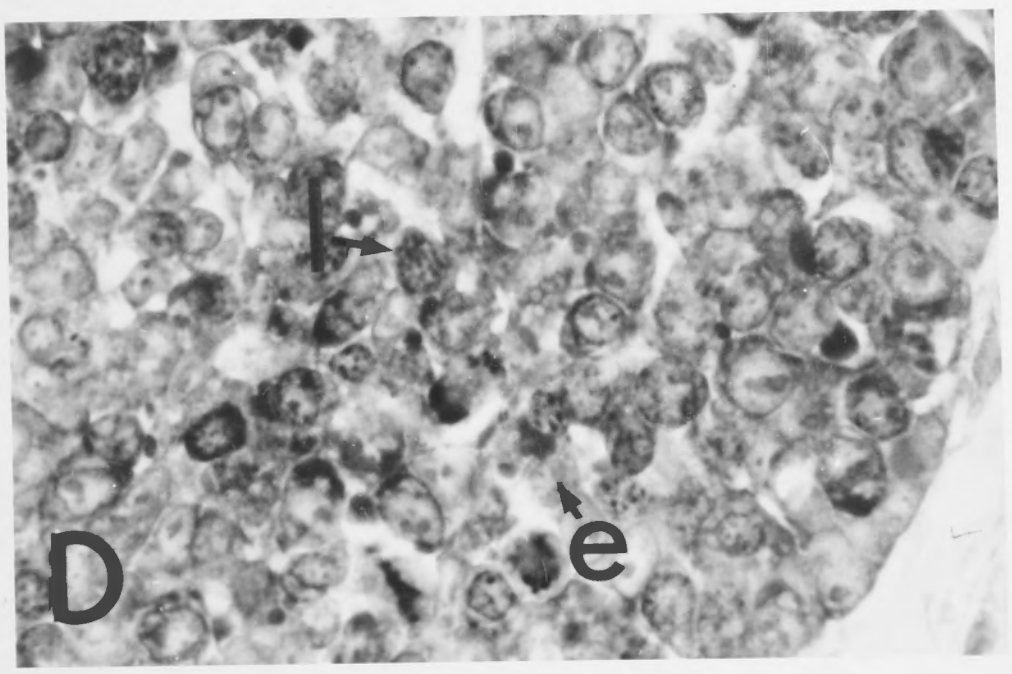
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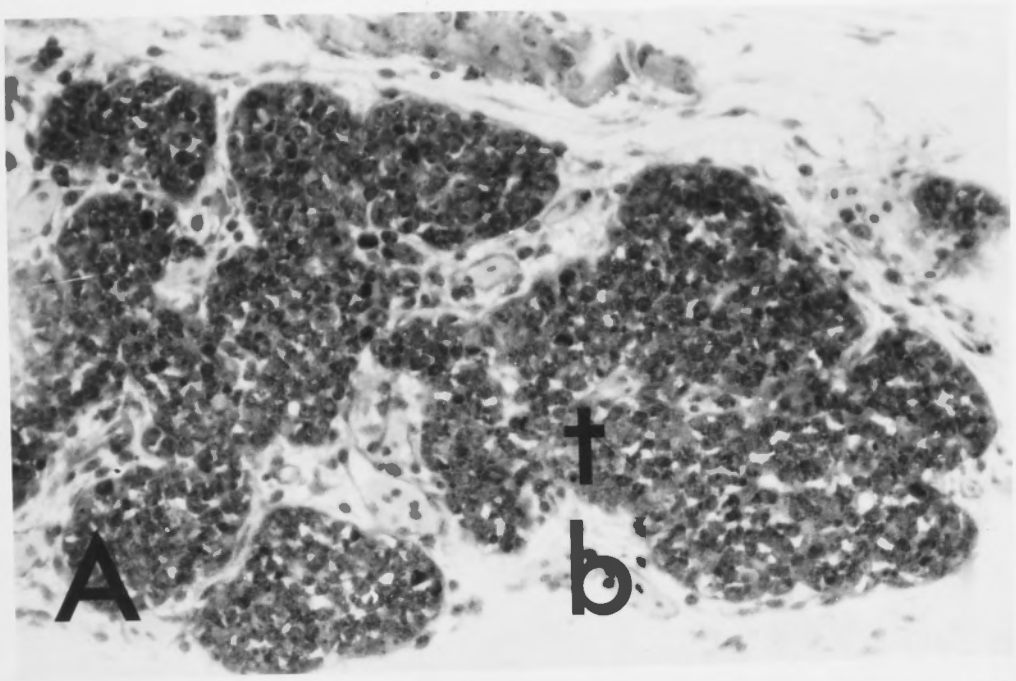
Fig. 7.13A-D Transverse sections from the thymus of a 12 day chick embryo inoculated at 6 days with adult allogeneic blood and from the thymus of normal embryos of the same age.

Fig. 7.13A A thymic lobe (t) from a normal 12 day embryo surrounded by connective tissue containing small blood vessels (b).
Azure II/methylene blue Magnification 250 x.

Fig. 7.13B A small proliferative lesion (p) within the thymus (t) of a 12 day embryo inoculated at 6 days with adult allogeneic blood. (cf) marks the surrounding connective tissue.
Azure II/methylene blue Magnification 250 x.

Fig. 7.13C At high magnification, the normal 12 day thymus is seen to contain large numbers of developing lymphoid cells (l). Small blood vessels (b) are found in the surrounding connective tissue but are not seen within the thymic lobe.
Azure II/methylene blue Magnification 1,000 x.

Fig. 7.13D The lymphoid cells in the thymus of a 6 day recipient are less numerous and larger in diameter (l). Epithelial cells (e) can be identified at the edge of the lobule. The proliferative lesion (p) consists of a small accumulation of large, pale staining cells.
Azure II/methylene blue Magnification 1,000 x.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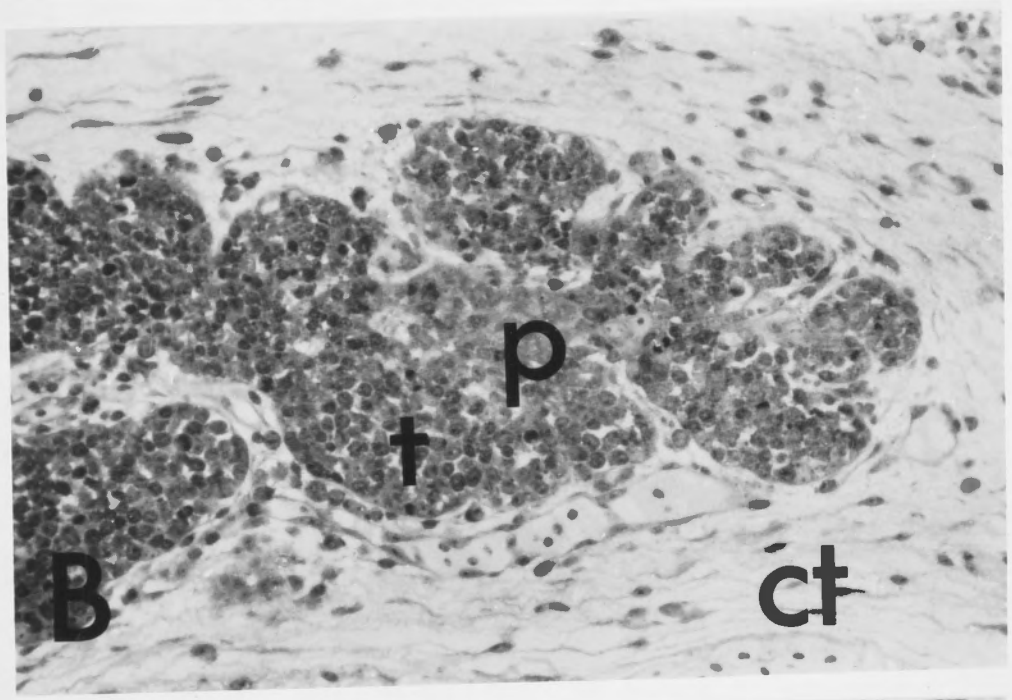
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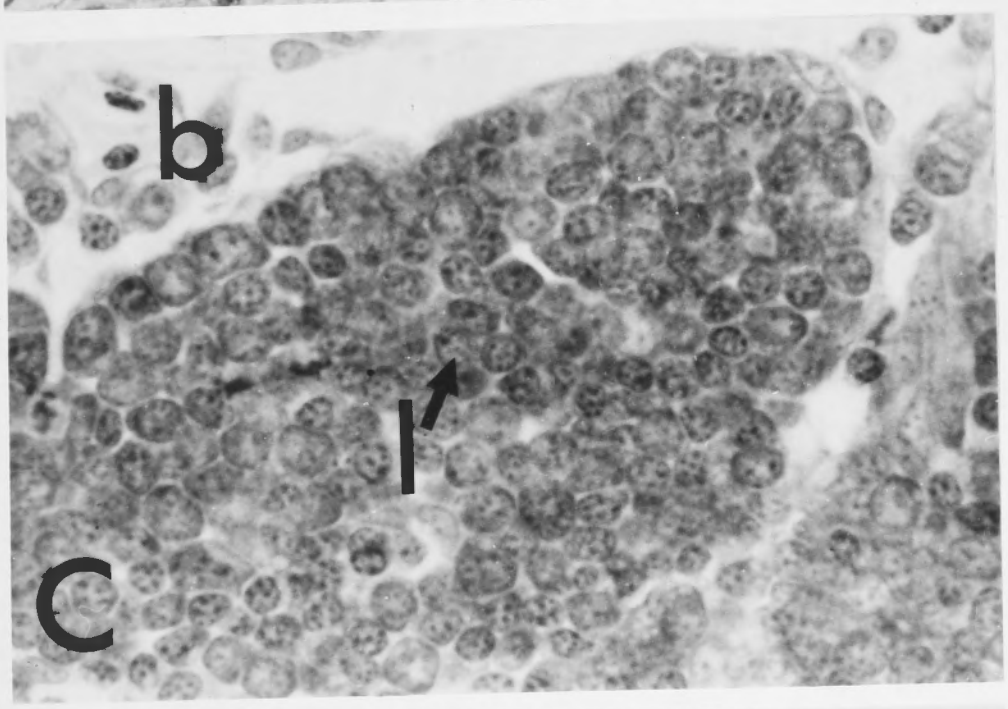
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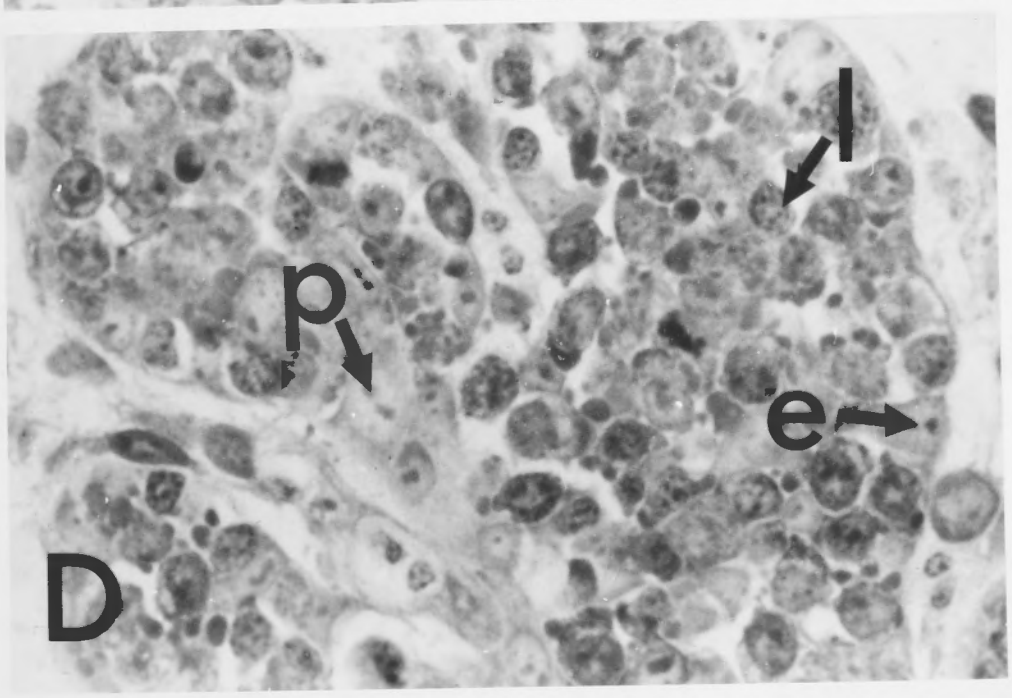
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entiation. Lymphoid cells were less numerous and larger in diameter than those seen in the normal thymus at the same age.

8 day recipients

Lymphopoietic activity in the thymus of normal embryos had further increased by 14 days (fig. 7.14A, fig. 7.14C). In experimental animals, however, which had been inoculated at day 8 with adult allogeneic blood, the lobular structure of the thymus was disrupted by the presence of proliferating reticular cells throughout the tissue (fig. 7.14B). Small clusters of thymocytes (fig. 7.14D) were separated by accumulations of these proliferating cells. All of the 9 embryos examined in this group had similar proliferative lesions within the thymus.

10 day and 14 day recipients

By the 16th day of incubation the cortex and the medulla of the thymus have differentiated although the 2 regions are not clearly delineated (fig. 7.15A, fig. 7.15C). Developing Hassall's corpuscles can be seen within the medulla and intense lymphopoesis continues in the cortex. In embryos of the same age, inoculated at day 10 with adult allogeneic blood, large proliferative lesions had developed in the thymus (fig. 7.15B, fig. 7.15D). They usually occurred in the medulla and extended into the cortex which still contained developing lymphocytes. In some areas these focal lesions of proliferating reticular cells became necrotic in the centre. Similar lesions were present in the thymus of all 10 day and 14 day recipients. The size of the lesion, however, was very variable and not all thymic lobes were affected.

These experiments showed that the type of pathological change which occurred in the thymus during a GVHR depends on the age of the recipient. A GVHR in very young recipients appears to result in a depression of lymphopoiesis in the thymus, while in older embryos proliferative lesions develop. A group of inbred embryos was used in a further experiment to determine whether the depression of lymphopoiesis in the thymus of very young embryos was specifically due to the GVHR.

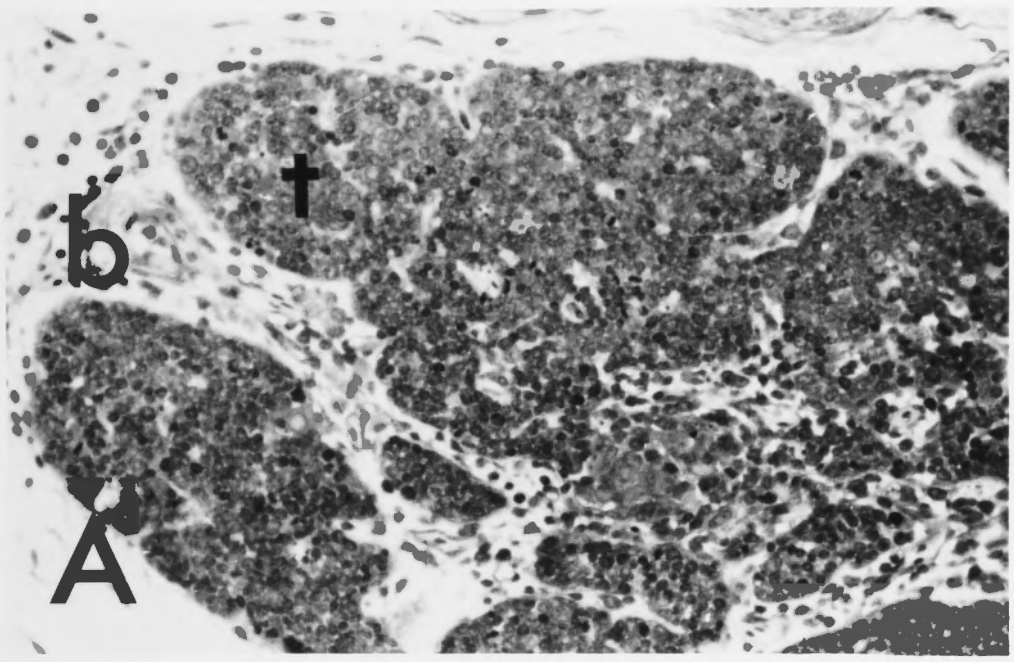
Fig. 7.14A-D Transverse sections from the thymus of a 14 day chick embryo inoculated at 8 days with adult allogeneic blood and from the thymus of a normal embryo of the same age.

Fig. 7.14A A thymic lobe (t) from a normal 14 day embryo surrounded by connective tissue containing small blood vessels (b).
Azure II/methylene blue Magnification 250 x.

Fig. 7.14B Islands of proliferating reticular cells (p) within the thymus of a 14 day embryo inoculated at 8 days with adult allogeneic blood. The proliferating cells have disrupted the normal pattern of lymphoid development within the thymic lobule (t). (ct) marks the connective tissue layer.
Azure II/methylene blue Magnification 250 x.

Fig. 7.14C A normal 14 day thymus at higher magnification to show the larger number of developing lymphoid cells (l) in the thymus at this stage. Thymic epithelial cells (e) can be identified at the edges of the lobule.
Azure II/methylene blue Magnification 1,000 x.

Fig. 7.14D Developing lymphoid cells (l) in the thymus of the 8 day recipient are divided into small clusters between the accumulations of primitive reticular cells (p).
Azure II/methylene blue Magnification 1,000 x.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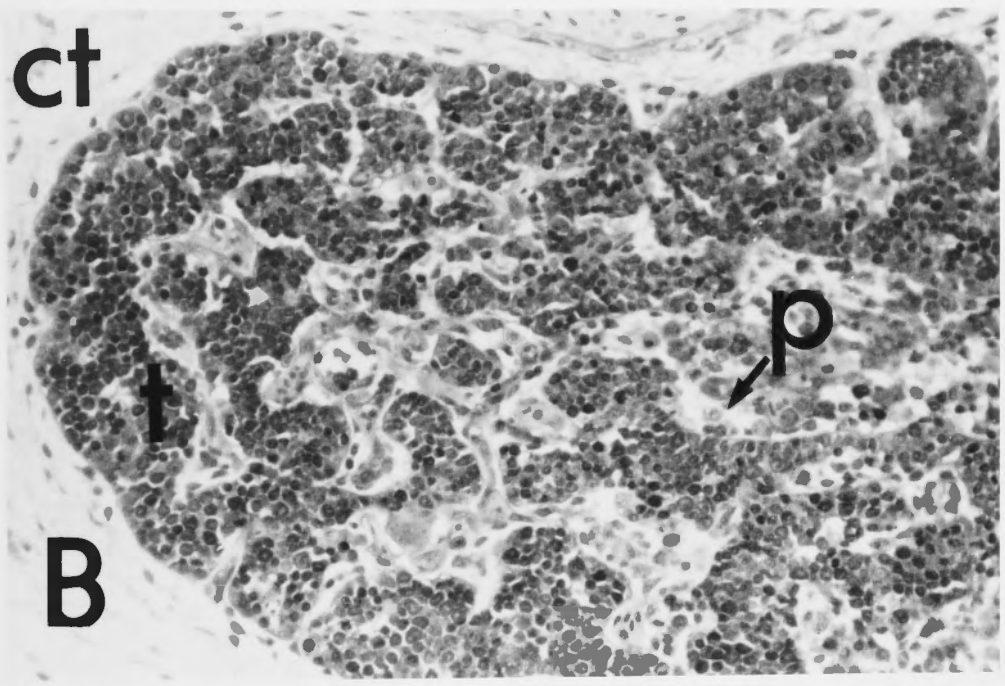
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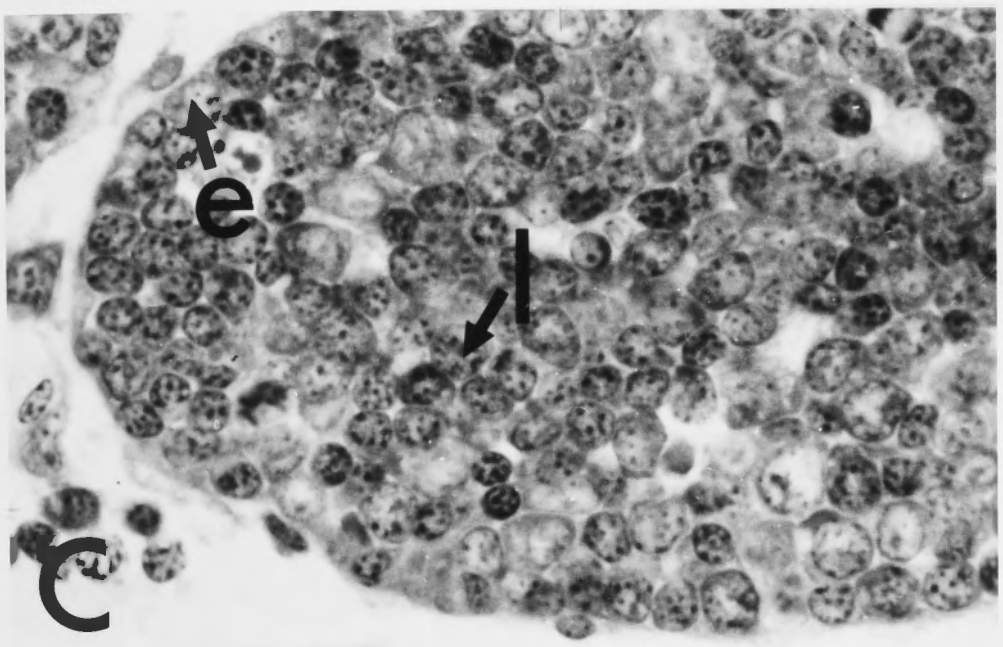
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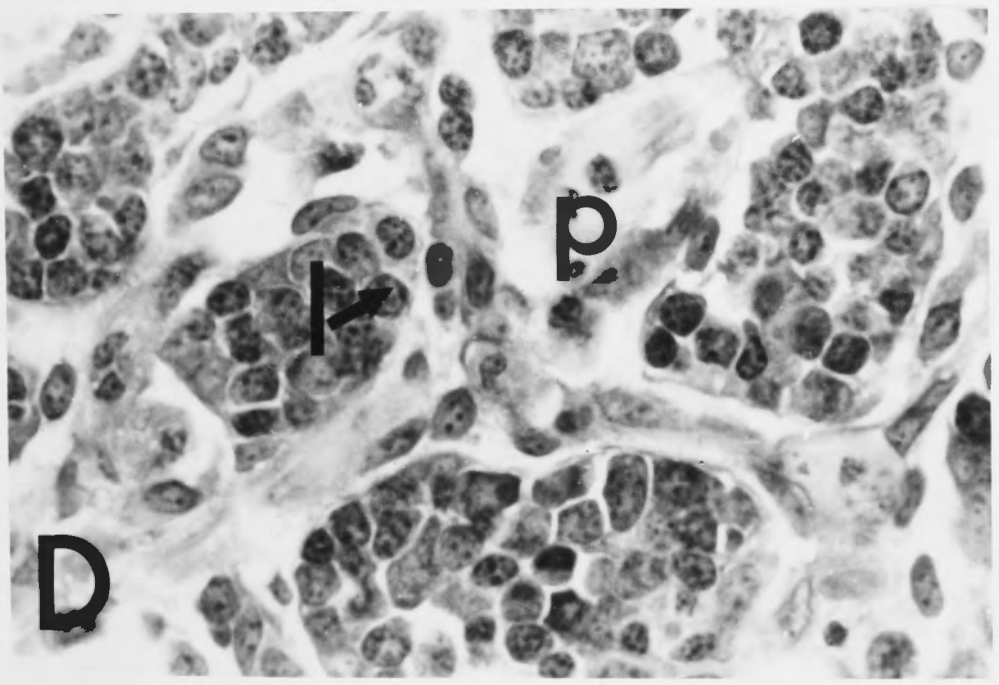
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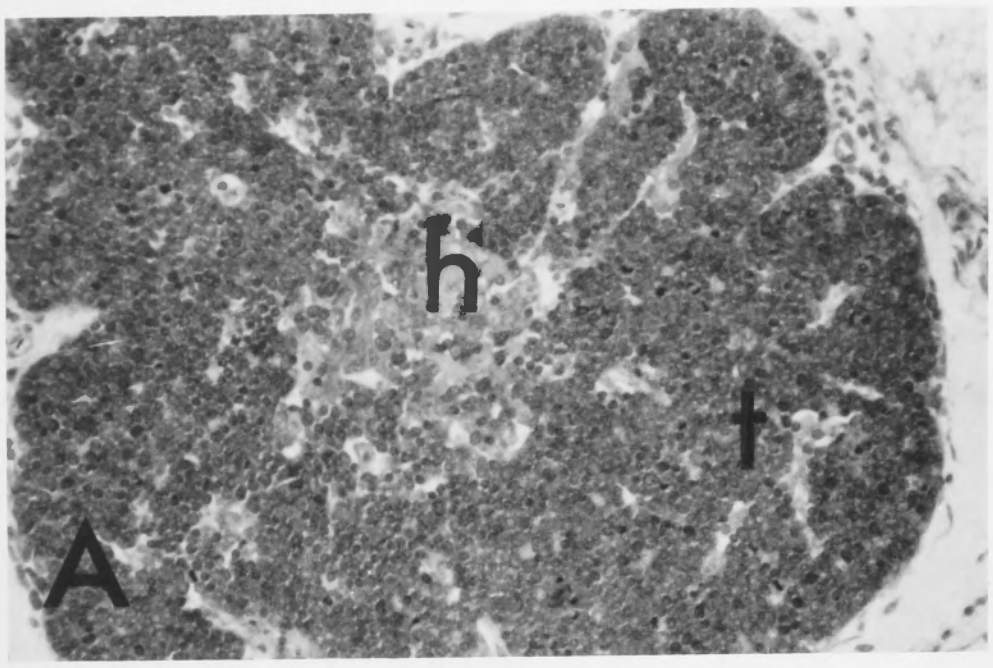
Fig. 7.15A-D Transverse sections from the thymus of a 16 day chick embryo inoculated at 10 days with adult allogeneic blood and from the thymus of normal embryos of the same age.

Fig. 7.15A A normal thymic lobe (t) from a 16 day old embryo in which an outer cortex and an inner medulla have now developed. Developing Hassall's corpuscles (h) are found in the medulla.
Azure II/methylene blue Magnification 250 x.

Fig. 7.15B A large proliferative lesion (p) within the thymus (t) of a 16 day old embryo inoculated at 10 days with adult allogeneic blood.
Azure II/methylene blue Magnification 250 x.

Fig. 7.15C A detail from the section in fig. 7.15A to show the development of lymphoid cells (l) within the normal 16 day thymus.
Azure II/methylene blue Magnification 1,000 x.

Fig. 7.15D A detail from the edge of the proliferative lesion in fig. 7.15B. Large, pale staining reticulum cells of the lesion are interspersed in this area with developing lymphoid cells (l).
Azure II/methylene blue Magnification 1,000 x.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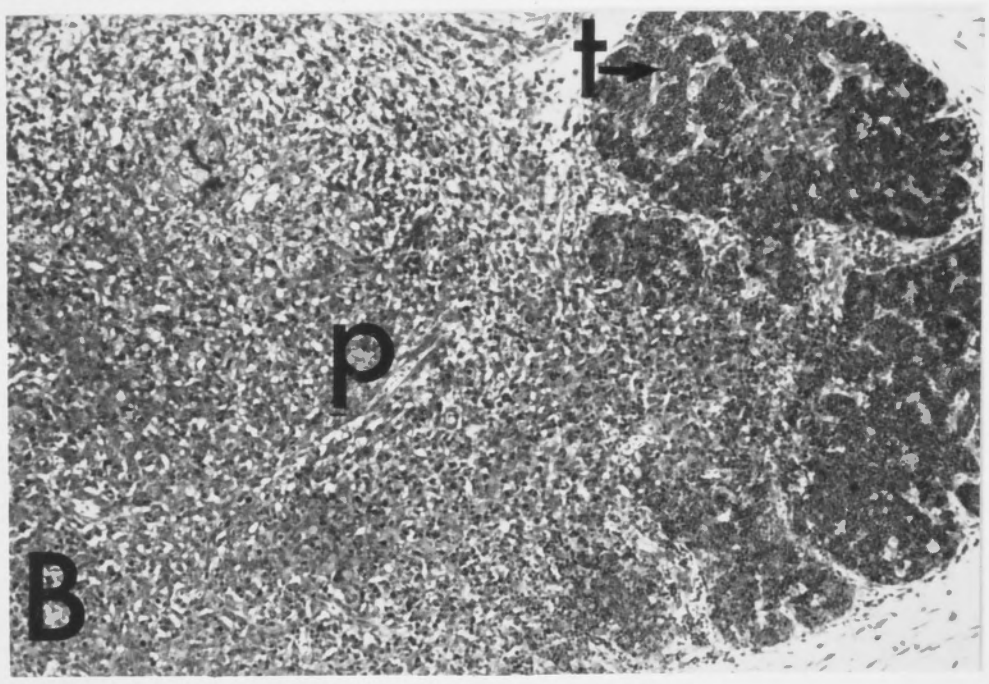
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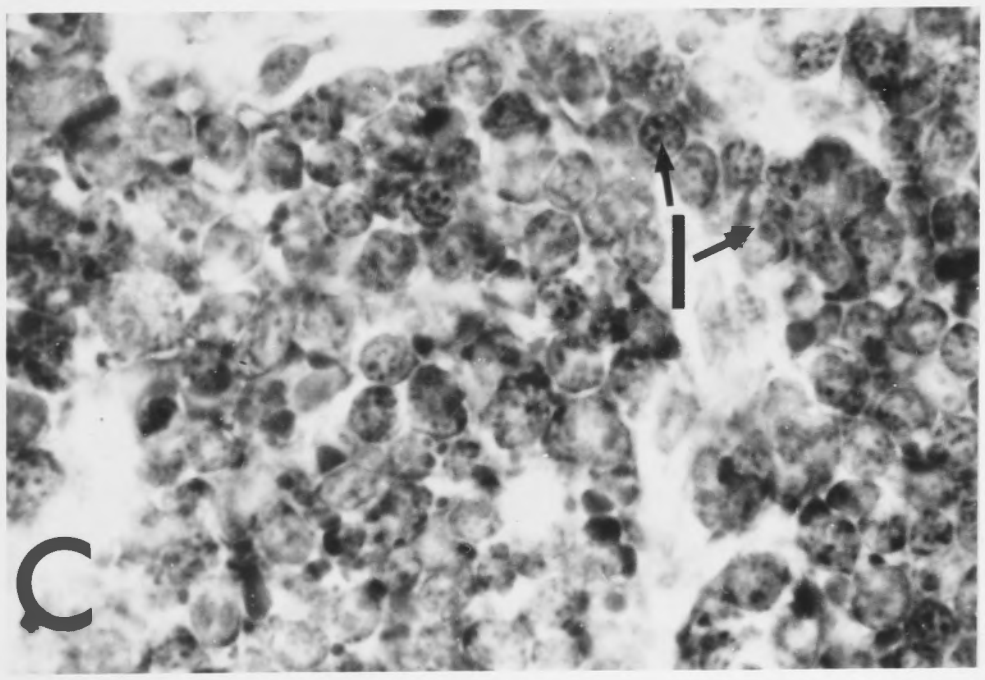
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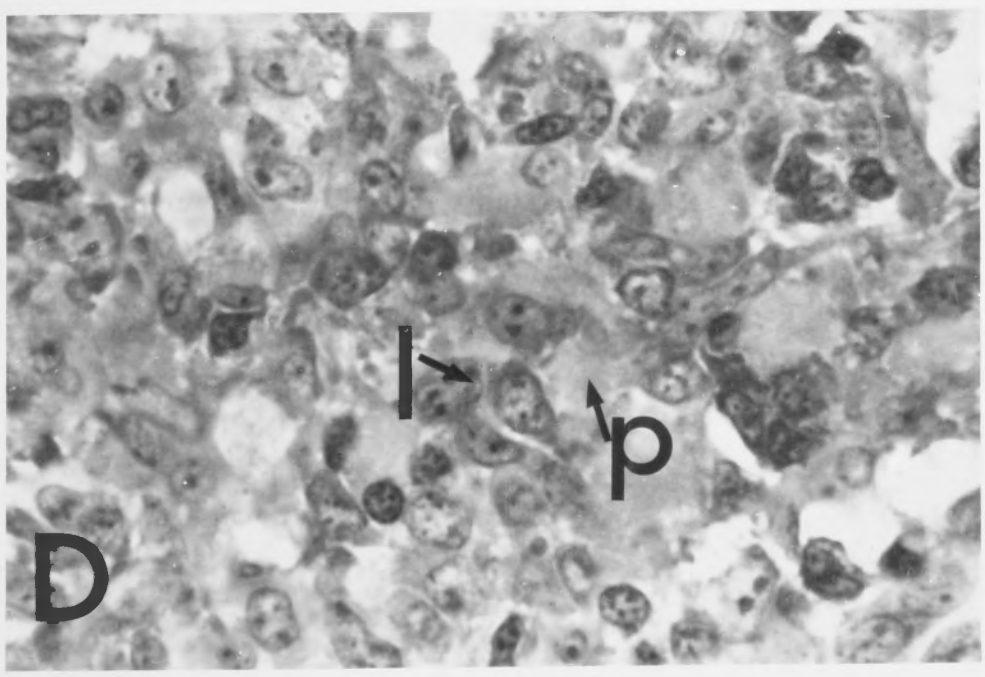
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The effect of allogeneic and syngeneic blood on the development of the thymus of 6 day recipients

Six day old CC or AA chick embryos were inoculated on the CAM with 0.1 ml of a 1:4 dilution of adult AA blood in Alsever's solution. Six days later the thymus was removed and examined by light microscopy.

Many of the CC embryos inoculated with adult allogeneic blood developed superficial haemorrhages similar to those described in chapter 4. The thymus from an embryo with only a few slight haemorrhages is shown in fig. 7.16A and fig. 7.16B. Lymphopoiesis in the thymus is depressed. At high magnification (fig. 7.16B) large, darkly staining cells which resemble the large lymphocytes described in the thymus by Venzke (1952) are interspersed among paler cells which appear to derive from the thymic epithelium.

Figs. 7.16C and 7.16D are taken from the thymus of another embryo which had severe haemorrhages. The poor cellularity of the thymic lobe is apparent. At higher magnification (fig. 7.16D) many of the pale cells within the lobe show degenerative changes.

In contrast, the inoculation of syngeneic adult blood into 6 day old AA embryos appeared to have no effect on the development of the thymus. At 12 days the thymus in these recipients showed intense lymphopoiesis (fig. 7.16E and 7.16F) comparable to that which occurs in normal randomly bred embryos of the same age (fig. 7.13A and 7.13C).

It was concluded that the inoculation of adult allogeneic blood into young chick embryos specifically depresses lymphopoiesis in the thymus. This effect appeared to be more severe in inbred embryos than in randomly bred recipients.

THE BONE MARROW

Normal development

The development of the marrow cavity in the long bones of the chick embryo has been described by Fell (1925). Blood vessels invade the cartilage matrix carrying osteoblasts and strands of connective tissue into the developing marrow cavity as they advance. A delicate reticular framework then forms within the cavity (Sabin and Miller, 1938).

Fig. 7.16A-F Transverse sections through the thymus to illustrate the pathological changes caused by the inoculation of allogeneic as compared to syngeneic adult blood into the 6 day embryo.

Fig. 7.16A Poor lymphoid development within the thymus (t) of a 12 day CC embryo inoculated at 6 days with adult allogeneic blood. (ct) marks the surrounding connective tissue.
Azure II/methylene blue Magnification 225 x.

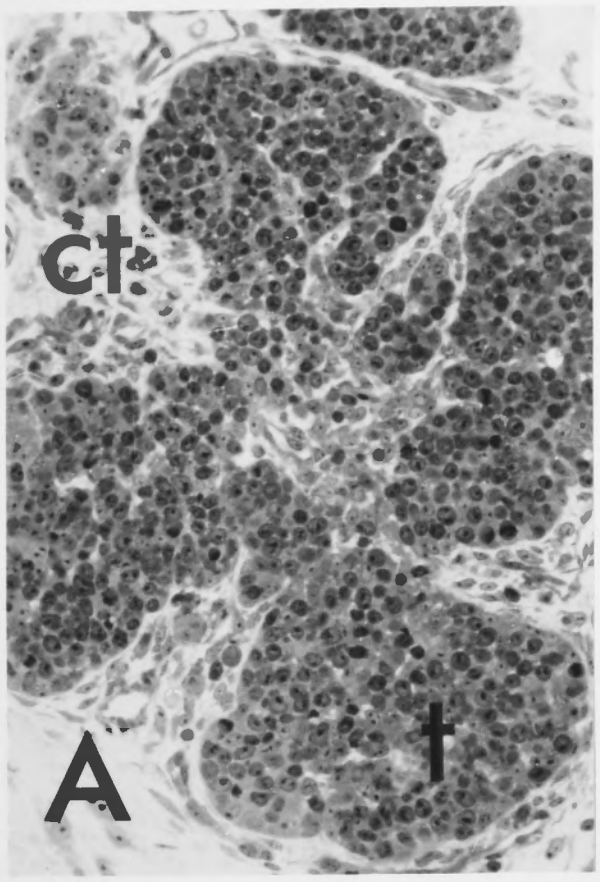
Fig. 7.16B Detail at a high magnification. Large darkly staining cells resembling large lymphocytes (l) are interspersed among more lightly staining cells of the thymic epithelium (e).
Azure II/methylene blue Magnification 1,700 x.

Fig. 7.16C Thymus from a 12 day old CC embryo which had developed severe haemorrhagic lesions after the inoculation of adult allogeneic blood at day 6. The poor cellularity of the thymus (t) is apparent. (ct) indicates the connective tissue layer.
Azure II/methylene blue Magnification 225 x.

Fig. 7.16D Detail at a high magnification. The cells of the thymus stain lightly and appear to have undergone degenerative changes (d).
Azure II/methylene blue Magnification 1,700 x.

Fig. 7.16E Thymic lobule (t) of normal appearance and surrounding connective tissue layer (ct) from a 12 day old AA embryo inoculated at 6 days with syngeneic adult blood.
Azure II/methylene blue Magnification 225 x.

Fig. 7.16F Detail to show the large number of developing lymphoid cells (l) and occasional mitotic figure (m). Thymic epithelial cells (e) can be identified at the edge of the lobule.
Azure II/methylene blue Magnification 1,700 x.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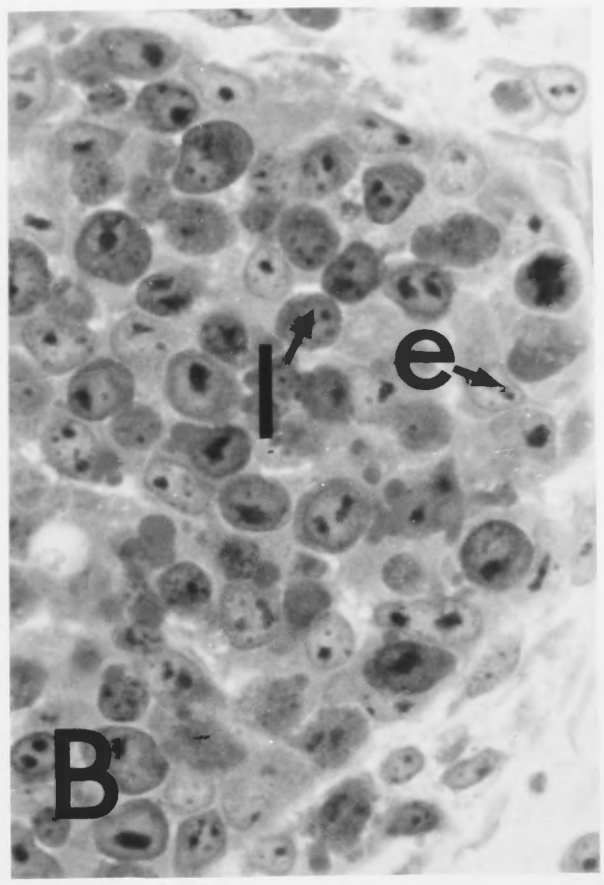
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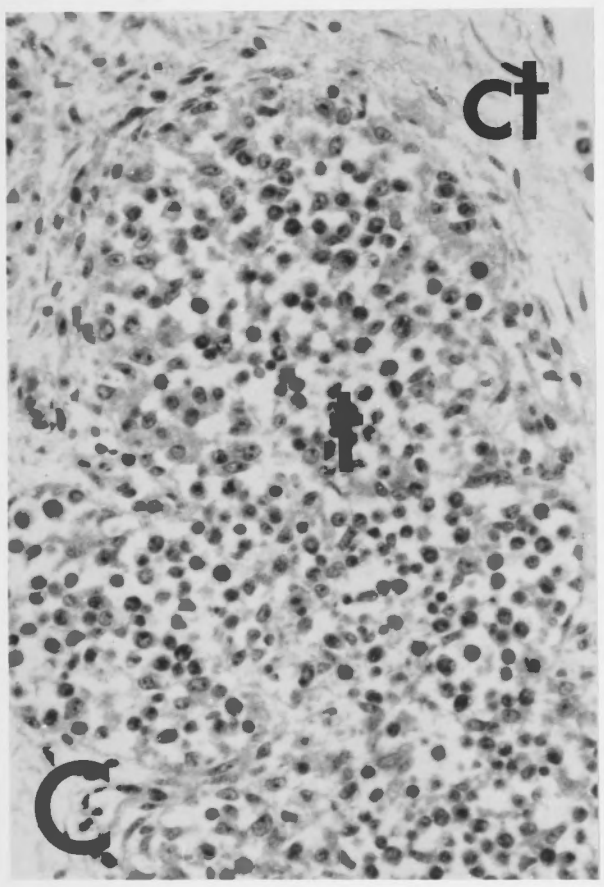
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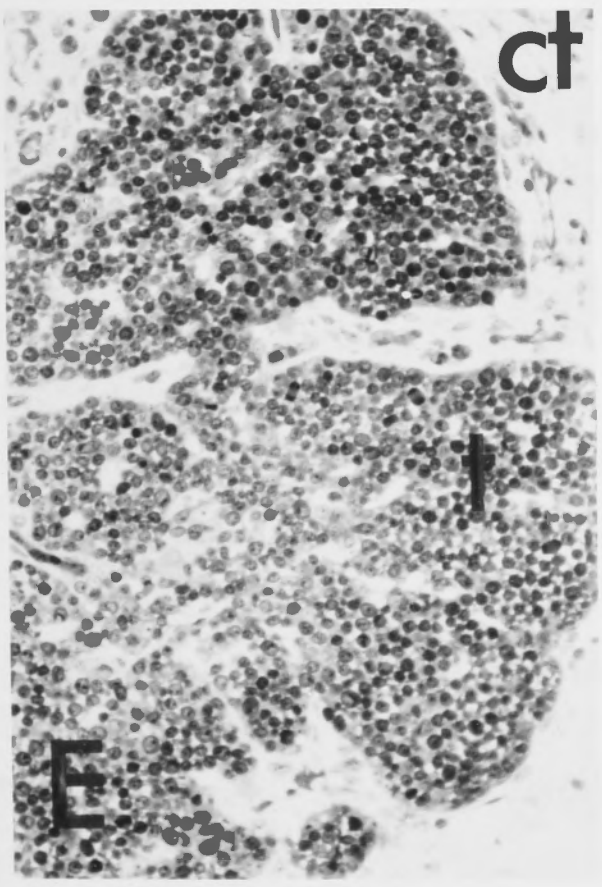
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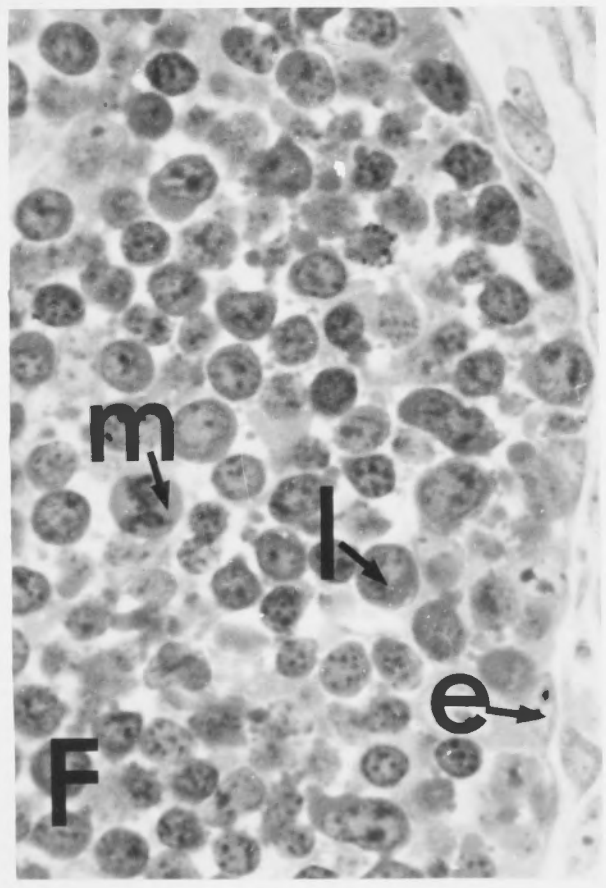
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Between 9 and 12 days eosinophilic leukocytes begin to develop extravascularly but definitive haemopoiesis does not commence until 10 or 12 days. Intravascular haemocytoblasts give rise to developing red cells while extravascular haemocytoblasts give rise to granulocytes (Dantschakoff, 1909; Romanoff, 1960).

The bone marrow, like other developing haemopoietic organs, is invaded, during development by yolk sac derived stem cells (Moore and Owen, 1965). Although the time at which stem cells first migrate into this tissue has not been precisely established, some stem cells are present in the bone marrow by 12 days incubation (Moore and Owen, 1965).

Experimental results

The effect of a GVHR on the development of the bone marrow

The effect of a GVHR on the development of the bone marrow was examined in randomly bred chick embryos of different ages, inoculated on the CAM with 0.1 ml of a 1:1 dilution of adult AA whole blood in Alsever's solution. Six days after inoculation the tibia was removed and examined by light microscopy.

5, 6 and 8 day recipients

The bone marrow of normal 11 day (fig. 7.17A), 12 day (fig. 7.17C) or 14 day old chick embryos (fig. 7.17E) has not yet reached the intense haemopoietic activity seen in older animals. Developing granulocytic cells are scattered throughout the tissue, between the venous sinusoids, but few developing red cells were observed at this stage. The bone marrow from experimental animals of the same age, inoculated at 5 (fig. 7.17B), 6 (fig. 7.17D) or 8 days of age (fig. 7.17E) was similar to that of normal embryos, although in 6 or 8 day recipients, granulopoietic activity in the bone marrow appeared to have increased. No proliferative lesions were found in the bone marrow of any of these recipients.

10 day and 14 day recipients

In the normal bone marrow, an increase in erythropoietic activity within the venous sinusoids is first evident at 16 days (fig. 7.18A) and becomes intense in the 20 day embryo (fig. 7.18C). Developing granulocytic cells

Fig. 7.17A-F Longitudinal sections through the tibia of normal 11 to 14 day old chick embryos and from experimental animals of the same age, inoculated at 5, 6 or 8 days with adult allogeneic blood.
Haematoxylin and eosin Magnifications 90 x.

Fig. 7.17A Normal 11 day bone marrow. Granulocytic cells are developing within the marrow cavity but there is little erythropoiesis.

Fig. 7.17B Bone marrow from an 11 day embryo inoculated at 5 days with adult allogeneic blood. There is less granulocytic activity than in the normal 11 day bone marrow.

Fig. 7.17C and fig. 7.17D Normal 12 day (fig. 7.17C) and 14 day (fig. 7.17D) bone marrow. The cells within the marrow are again predominantly developing granulocytes.

Fig. 7.17E and fig. 7.17F Bone marrow from a 12 day (fig. 7.17E) and 14 day (fig. 7.17F) chick embryo inoculated at 6 and 8 days, respectively with adult allogeneic blood. Granulocytic activity in the bursa has increased more than in normal embryos of the same age. There are no proliferative lesions.


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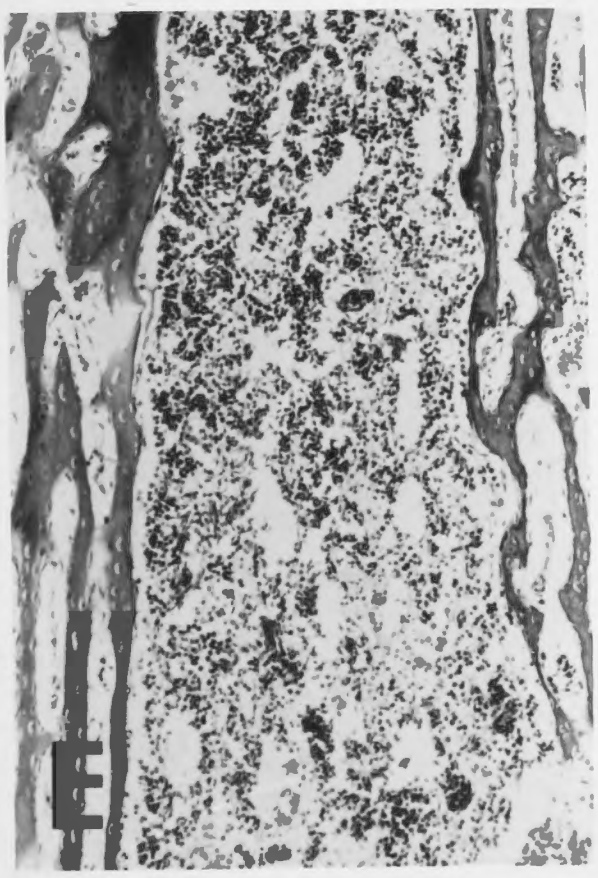
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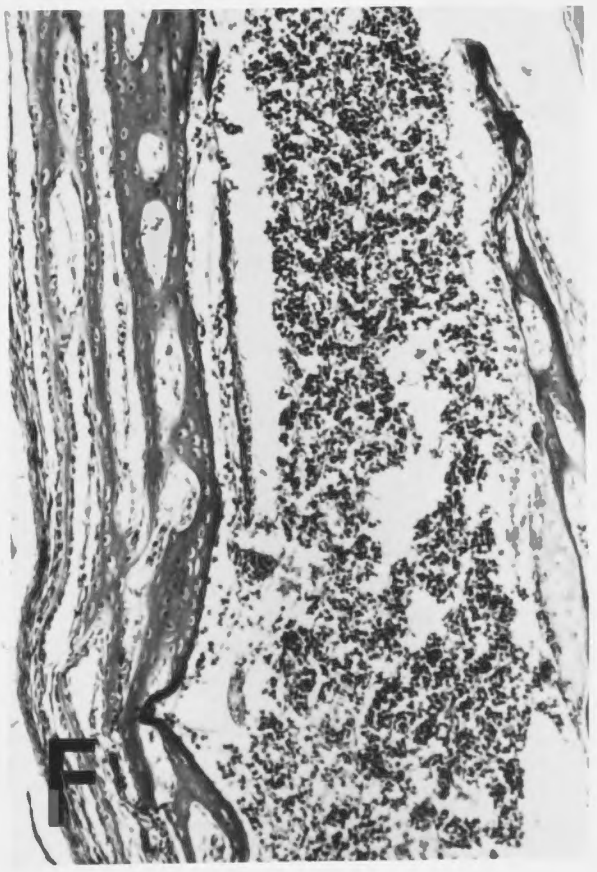
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Fig. 7.18A-D Longitudinal sections through the tibia of normal 16 and 20 day old chick embryos and from experimental animals of the same age inoculated at 10 or 14 days with adult allogeneic blood.
Haematoxylin and eosin Magnifications 90 x.

Fig. 7.18A Normal 16 day bone marrow. Erythropoiesis is beginning to increase within the marrow sinusoids.

Fig. 7.18B Bone marrow from a 16 day embryo inoculated at 10 days with adult allogeneic blood. A large proliferative lesion (p) has developed within the marrow and granulopoiesis appears to have increased in the surrounding tissue.

Fig. 7.18C Normal 20 day bone marrow. Intense erythropoietic activity is now seen in the marrow sinusoids while granulocytic cells differentiate in the surrounding tissue.

Fig. 7.18D Bone marrow from a 20 day old embryo inoculated at 14 days with adult allogeneic blood. Granulopoiesis appears to be augmented. A large focal accumulation of proliferating cells (p) similar to that in fig. 7.18B has developed.

Fig. 7.18E A detail from the proliferative lesion in fig. 7.18D. The cells are light-staining and similar to those described in proliferative lesions in the thymus and bursa.
Haematoxylin and eosin Magnification 900 x.


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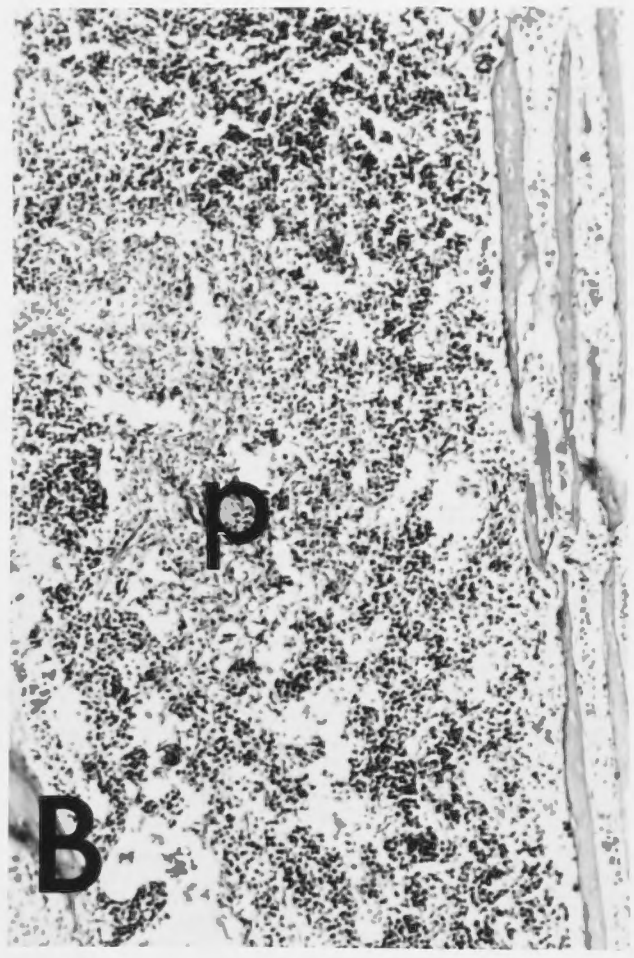
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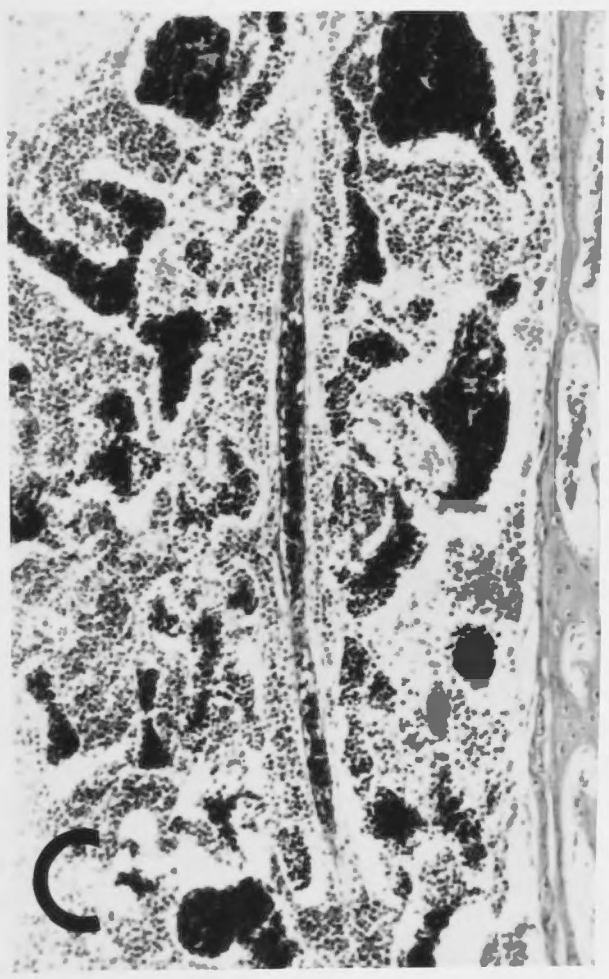
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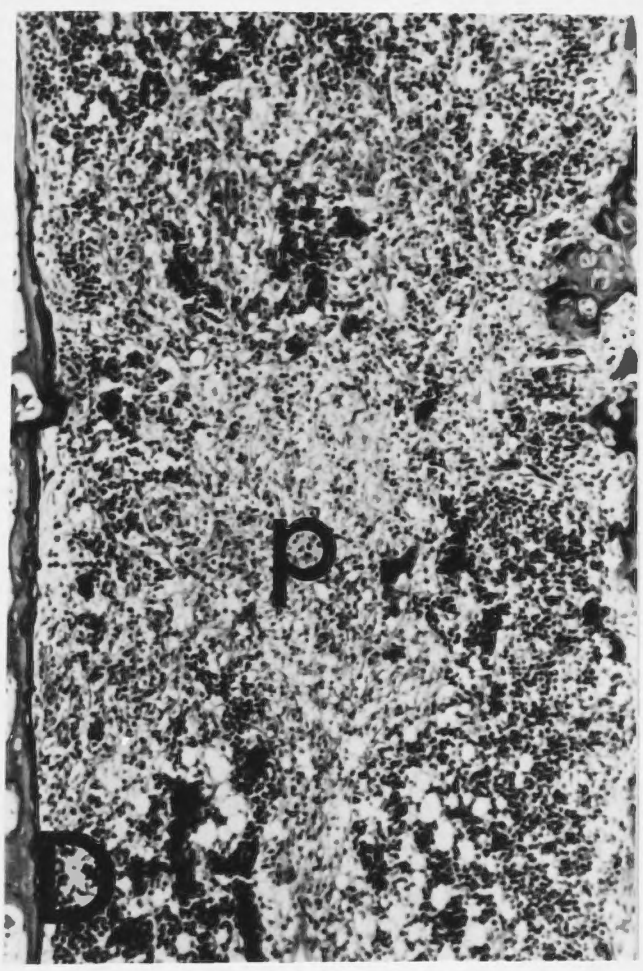
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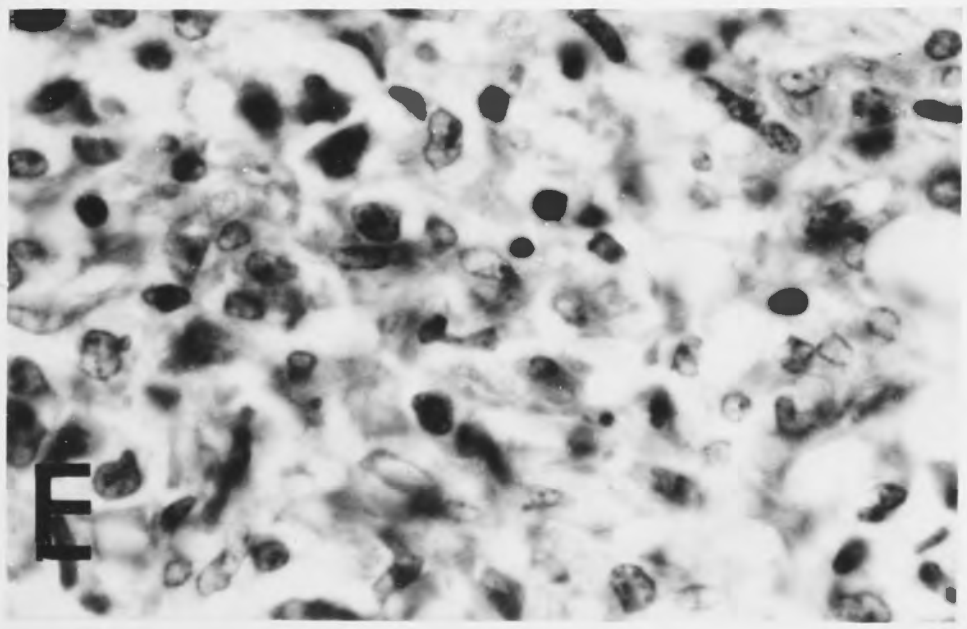
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are scattered throughout the rest of the bone marrow. The marrow from experimental animals of the same age, inoculated at 10 days (fig. 7.18B) or 14 days (fig. 7.18D) with adult allogeneic blood showed a large increase in granulocytic activity. The number of developing red cells, however, appeared to have decreased. Proliferative lesions were observed in 5 of the 10 animals inoculated at 10 days and in 9 of the 12 animals inoculated at 14 days. These lesions consisted of focal accumulations of proliferating cells which occurred throughout the marrow cavity. These cells were lightly staining (fig. 7.18E) and resembled primitive reticulum cells similar to those already described in proliferative lesions in the bursa (fig. 7.3D, 7.8A) and in the thymus (fig. 7.17B, 7.17D).

These experiments indicate that, as in the case of the bursa and thymus, the nature of pathological changes in the bone marrow during a GVHR, depends on the age of the recipient. Six or 8 day recipients show an increase in granulopoiesis in the bone marrow, while older embryos develop proliferative lesions.

Discussion

The nature of pathological changes which occur in the thymus, the bone marrow and the bursa during a GVHR in the chick embryo has been shown to depend on the age of the recipient. Very young recipients show lymphoid aplasia which is more marked in the thymus and the bursa than in the bone marrow. The depression of lymphopoiesis in these tissues appears to be due to a destruction of progenitor cells in the yolk sac during early embryonic life (chapter 6). This presumably prevents the migration of yolk sac derived stem cells into the anlagen of intra-embryonic haemopoietic tissues causing lymphoid aplasia in these organs. Granulopoiesis does not appear to be as severely affected as lymphopoiesis and, in certain cases, may even be augmented while lymphoid development is depressed.

Descriptions of graft-versus-host reactions in the chick embryo have usually concerned the development of proliferative lesions rather than lymphoid aplasia. Fennell (1966), however, noted that bursal weight was

significantly depressed in 20 day chick embryos grafted 10 days previously with adult thymus or spleen. Thus decreased follicle formation in the bursa appears eventually to affect bursal weight, although experiments described in this chapter, in agreement with Seto (1968), did not show a decrease in bursal weight, 6 days after inoculation.

Proliferative lesions were observed in the thymus, bursa and bone marrow only in older embryos. These lesions were similar to those which have previously been described in the bursa (Lafferty and Jones, 1969), spleen (Biggs and Payne, 1961a, b; DeLanney et al., 1962), bone marrow and thymus (Simonsen, 1957; Boyer, 1960; Lafferty and Jones, 1969).

The time when proliferative lesions first develop in the thymus, bone marrow and bursa could be closely correlated with the time when yolk sac derived stem cells first migrated into these tissues (table 7.1).

Table 7.1

Relationship between the appearance of haemopoietic stem cells in various haemopoietic tissues as determined by the experiments of Moore and Owen, and the time at which the introduction of donor cells first produced proliferative lesions during a GVHR.

	Tissue		
	Thymus	Bone marrow	Bursa
Time when haemopoietic stem cells first enter the tissues	8 days	about 12 days	14 days
Reference	Moore & Owen (1967) Owen & Ritter (1969)	Moore & Owen (1965)	Moore & Owen (1966)
Time when inoculation of donor cells first produces a proliferative lesion	about 8 days	10 to 14 days	14 days

The correlation between these two events supports the suggestion that proliferative lesions can only develop if the tissue contains yolk sac derived stem cells. In relation to this suggestion, it is interesting that DeLanney and coworkers (1962) observed a striking increase in haemocytoblasts in the spleen and the CAM shortly after the inoculation of donor cells and before proliferative lesions appeared in these tissues.

The experiments described in chapters 6 and 7 suggest that the pathogenesis of a GVHR in the chick embryo involves 2 types of interaction between adult allogeneic lymphocytes and cells of the embryonic haemopoietic tissue. Stem cells which have migrated from the yolk sac into other developing haemopoietic organs may be stimulated to proliferate while primitive haemopoietic stem cells within the yolk sac appear to be destroyed or inactivated. Since the extent of lymphoid aplasia declines and proliferative lesions increase as the various haemopoietic organs are progressively invaded by stem cells, the pathological changes which occur during a GVHR closely reflect the state of development of these haemopoietic tissues.

CHAPTER 8

THE EFFECT OF SEQUENTIAL DONOR CELL INOCULATION ON THE PATHOGENESIS OF THE GRAFT-VERSUS-HOST REACTION

CHAPTER 8. THE EFFECT OF SEQUENTIAL DONOR CELL INOCULATION ON THE PATHOGENESIS OF THE GRAFT-VERSUS-HOST REACTION

Introduction

In chapter 7 it was suggested that adult allogeneic lymphocytes may interact in two different ways with haemopoietic stem cells. Primitive stem cells in the yolk sac appear to be destroyed or inactivated while yolk sac derived stem cells, which have migrated into the various intra-embryonic haemopoietic organs are stimulated to proliferate. On the basis of this hypothesis it may be postulated that pock formation on the CAM depends on the presence of yolk sac derived stem cells in the circulating blood.

It has been shown that a GVHR in very young chick embryos destroys haemopoietic stem cells in the blood islands of the yolk sac and the concomitant aplasia of intra-embryonic haemopoietic organs was thought to result from lack of colonization by yolk sac derived stem cells. If this interpretation is correct and if proliferative lesions depend on the presence of yolk sac derived stem cells either in the embryo's haemopoietic tissues or in the circulation, then inoculation of allogeneic donor cells some time after an early GVHR should not induce proliferative lesions. This was tested in the following experiments.

Experimental results

Experimental design

A GVHR was produced in very young embryos by 'early treatment' with adult blood. These embryos were then 'challenged' several days later with another inoculum of adult blood placed on the CAM to determine their pock forming ability.

Early treatment

Chick embryos were inoculated under the shell membrane which lines the air space (chapter 2) at 5 days and again at 6 days with 0.1 ml of a 1:1 dilution of adult AA blood in Alsever's solution. Control embryos were inoculated in a similar manner with 0.1 ml of Alsever's

Table 8.1

Pock formation on the CAM of chick embryos inoculated at 10 days incubation following an early treatment at 5 and 6 days with adult blood.

Embyro recipients	Early treatment 5 days and 6 days	Challenge at 10 days	Average pock count per membrane at 13 days ± standard error	t-Test probability
Randomly bred	Adult AA blood	Adult AA blood	3.6 ± 2.7	0.0008
Randomly bred	——	Adult AA blood	25.8 + 3.9	
Randomly bred	Adult AA blood	Adult donor 1*	5.4 ± 1.5	0.0080
AA	——	Adult donor 1*	16.9 ± 2.5	
AA	Adult AA blood	Adult donor 2*	18.6 ± 3.3	0.7079
AA	——	Adult donor 2*	20.3 ± 2.7	
AA	——	Adult AA blood	2.1 ± 0.7	0.0002
AA	——	Adult donor 1*	16.9 ± 2.5	

*Blood from adult randomly bred donors which reacted against AA recipients.

solution.

Challenge

At 10 days these recipients were inoculated on the CAM (chapter 2) with 0.1 ml of a 1:1 dilution of adult blood in Alsever's solution. The blood used for this inoculation was taken either from adult AA donors or from adult randomly bred donors whose blood was known to produce pocks on the CAM of normal 10 day old AA chick embryos.

Three days after this last inoculation, the eggs were opened and the number of pocks on the CAM was counted with the aid of a dissecting microscope. Each embryo was then examined for signs of haemorrhage and the bursa, thymus and yolk sac were fixed and embedded for light microscopy. The mortality which occurredin each experiment was also noted.

The effect of early treatment with allogeneic blood on pock formation

As shown in table 8.1, randomly bred chick embryos inoculated at day 5, day 6 and day 10 with adult AA blood had a very low average pock count at 13 days. Many of the embryos in this experimental group (6/8) had no pocks at all on the CAM, although one embryo was found with a high pock count (21 pocks). In comparison, randomly bred chick embryos given an inoculation of adult AA blood at 10 days without early treatment had a high pock count 3 days later. The difference between the average pock count on the CAM in these 2 groups was found to be statistically significant ($p<0.01$).

One explanation of the depression of pock forming ability after an early treatment with allogeneic blood might be that a repopulation of the embryo with AA cells of the early inoculum renders it unable to form pocks in response to another inoculation of blood from the same AA donor. In order to exclude this possibility, embryos were given an early treatment with adult AA blood, followed by a challenge with blood from an adult, randomly bred donor. As shown in table 8.1, although the inoculation of blood from this randomly bred donor produced many pocks on the CAM of 10 day old AA embryos, the same inoculation gave very few pocks when introduced onto the CAM of embryos

which had been previously inoculated at 5 and 6 days with adult AA blood. The depression in pock forming ability after an early GVHR thus did not appear to be due to the repopulation of the embryo with donor cells.

The effect of early treatment with syngeneic adult blood on subsequent pock forming ability

Experiments were also carried out in AA recipients to determine the specificity of the depression of pock forming ability. Young AA recipients inoculated at day 5 and day 6 with adult AA blood, were subsequently challenged with blood from a randomly bred donor known to react against AA recipients.

As shown in table 8.1, embryos which received an early treatment with syngeneic adult blood did not show a significant depression of pock forming ability in response to a subsequent challenge of allogeneic blood. The depression in pock forming ability observed in previous experiments thus appeared to be specifically caused by the early GVHR.

When adult AA blood was inoculated onto the CAM of 10 day old syngeneic recipients, very few pocks were formed (table 8.1) which reflects the high degree of inbreeding in this strain.

The effect of multiple inoculation on embryo survival

Embryos in each of the experimental groups described above were candled at intervals during incubation to determine the effect of multiple inoculation on survival. Very few randomly bred embryos inoculated at day 5 and day 6 with adult allogeneic blood before the challenge at 10 days for pock forming ability, survived until 13 days (fig. 8.1A). A control group, inoculated with adult allogeneic blood at 10 days without early treatment, however, had a much better survival rate (fig. 8.1A).

AA embryos generally have a much higher mortality rate than randomly bred embryos, due to the high degree of inbreeding. However, despite this, the mortality among AA recipients inoculated at 5 and 6 days with syngeneic adult blood before the challenge for pock forming ability was not lower than the mortality incurred by AA embryos which did

Fig. 8.1A The effect of multiple inoculations of adult blood on the survival of randomly bred chick embryos.

Group A. Randomly bred chick embryos given an early treatment with adult AA blood and then challenged at 10 days with adult AA blood.

Group B. Randomly bred chick embryos given an early treatment with adult AA blood and then challenged at 10 days with blood from a randomly bred adult donor.

Group C. Randomly bred chick embryos inoculated at 10 days with adult AA blood.

Fig. 8.1B The effect of a multiple inoculation of adult blood on the survival of inbred AA chick embryos.

Group A. Inbred AA chick embryos given an early treatment with adult AA blood and challenged at 10 days with blood from an adult randomly bred donor.

Group B. Inbred AA chick embryos inoculated at 10 days with blood from an adult randomly bred donor.

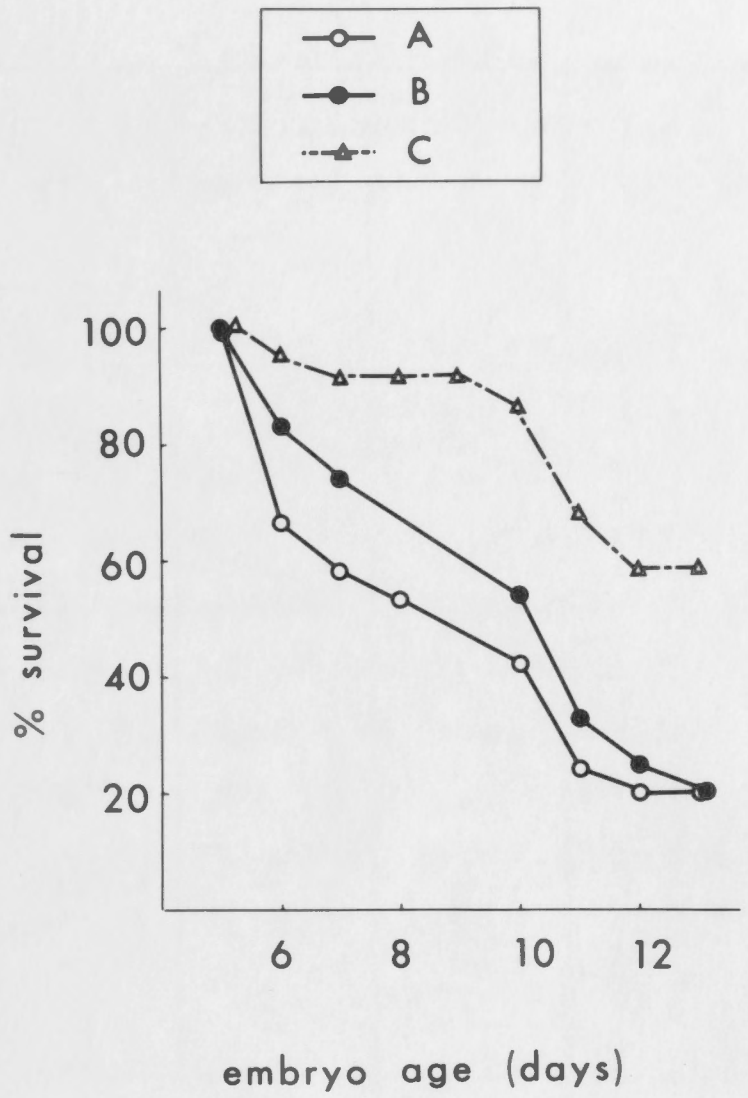


fig. 8.1 A

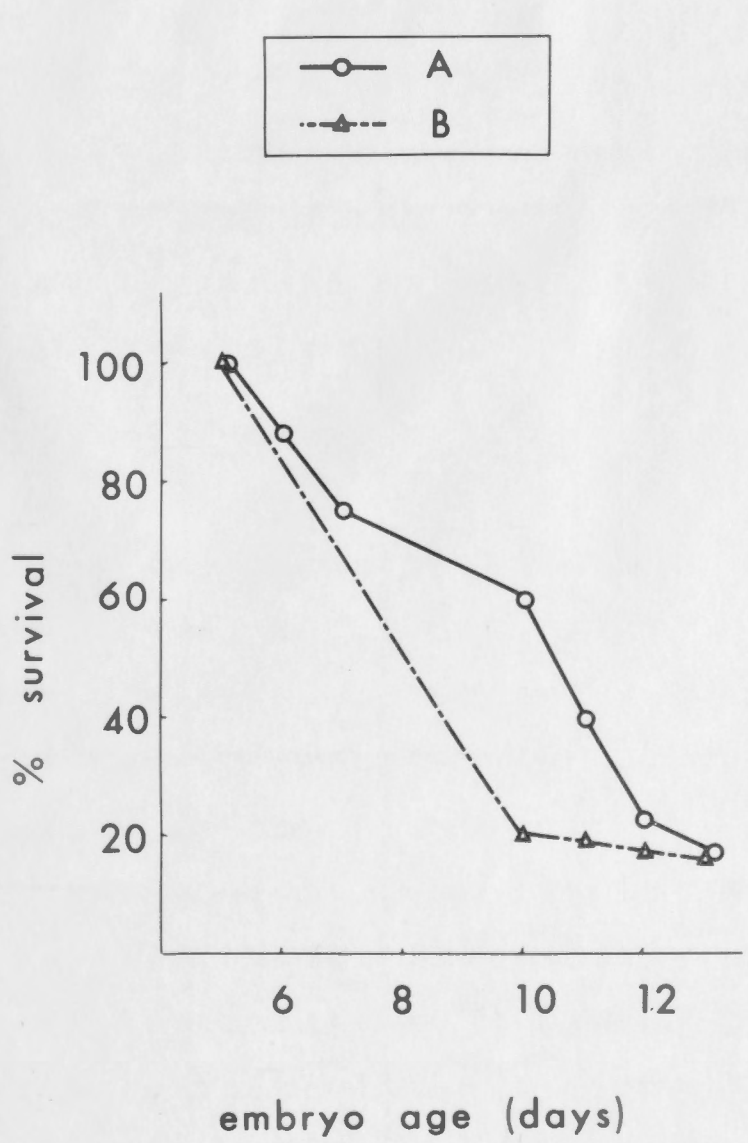


fig.8.1 B

Table 8.2

Development of a haemorrhagic condition in chick embryos given multiple inoculations with adult blood.

Embryo recipients	Early treatment at 5 and 6 days	Challenge at 10 days	Number of surviving recipients	Number of surviving recipients with haemorrhage
Randomly bred	Adult AA blood	Adult AA blood	8/45	7
Randomly bred	——	Adult AA blood	12/21	0
Randomly bred	Adult AA blood	Adult donor 1*	9/46	8
AA	——	Adult donor 1*	21/94	0
AA	Adult AA blood	Adult donor 2*	9/52	0
AA	——	Adult donor 2*	9/105	0

*Blood from adult randomly bred donors which reacted against AA recipients.

not receive the early treatment (fig. 8.1B). This indicates that the poor survival in randomly bred embryos is specifically related to the development of an early GVHR in these animals.

Development of haemorrhages after multiple inoculation

Surviving embryos from each of the experiments described above, were examined at 13 days for signs of haemorrhages. As shown in table 8.2, many of the randomly bred embryos which had been inoculated at day 5 and day 6 with adult allogeneic blood before the challenge for pock forming ability at 10 days had haemorrhages on the body surface and in the CAM by 13 days. An embryo with severe haemorrhages is illustrated in fig. 8.2A. The yolk sac from this embryo (fig. 8.2B) appeared very fragile and folds were very poorly developed. Large areas of the membrane are transparent which suggests that the epithelial cells are no longer absorbing yolk.

As shown in table 8.2, AA embryos which were inoculated at day 5 and day 6 with adult syngeneic blood before the challenge at day 10 had no haemorrhages at 13 days. Similarly, embryos inoculated at day 10 with adult blood, without early treatment did not develop haemorrhagic lesions. Since the haemorrhagic lesions only developed in embryos inoculated at day 5 and day 6 with adult allogeneic blood, they appear to be specifically related to the development of an early GVHR.

Changes in the yolk sac after multiple inoculation of adult blood

In each of the experiments described in this chapter, the yolk sac was removed at 13 days and fixed for light microscopy. Sections were cut along the length of a yolk sac fold and examined in order to determine the effect of multiple inoculation on the blood islands.

In the yolk sac fold of a normal 13 day old randomly bred chick embryo (fig. 8.3A, fig. 8.4A) the central arterial vessel is surrounded by a plexus of periarterial venous capillaries in which intense haemopoiesis is taking place. The developing cells within the vascular lumen are tightly packed together and stem cells are closely apposed to the endothelium.

Fig. 8.2A A 13 day old randomly bred chick embryo with severe haemorrhages after inoculation at day 5, day 6 and day 10 with 0.1 ml of diluted adult AA blood.

Fig. 8.2B The yolk sac from the embryo shown in fig. 8.2A. The yolk sac folds are poorly developed and large areas of the membrane are transparent suggesting that epithelial cells are no longer absorbing yolk.

Fig. 8.2 A

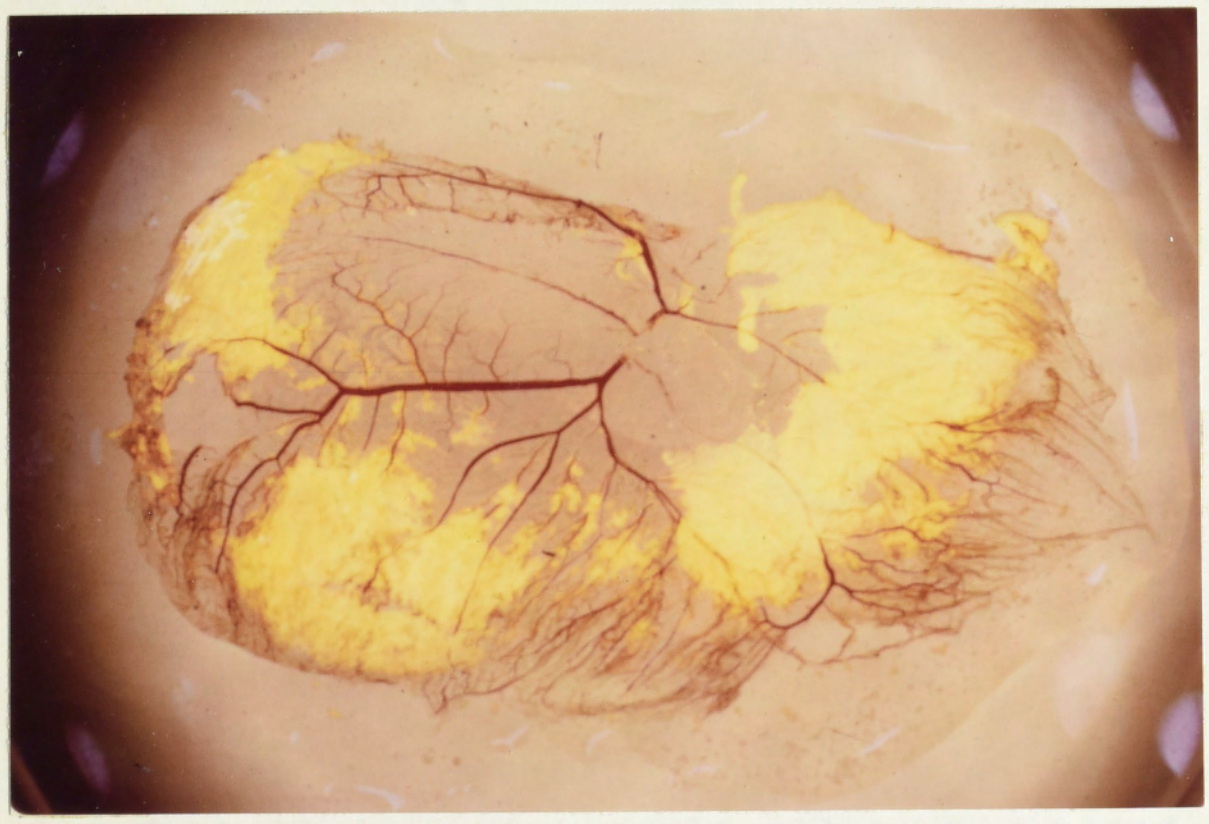


Fig. 8.2 B

The effect of an early GVHR on subsequent development of the yolk sac is evident in sections from embryos inoculated at day 5 and day 6 with adult AA blood, followed with a challenge of either adult AA blood (fig. 8.3C) and fig. 8.4C) or blood from an adult randomly bred donor (fig. 8.3E and fig. 8.4E). In these embryos, blood islands of the yolk sac are severely depleted. In many recipients only a few haemopoietic stem cells remained in the venous capillaries, clustered near the vessel walls (fig. 8.3C and fig. 8.4C). In several other cases the venous capillaries in the yolk sac fold had become congested with mature erythrocytes (fig. 8.3E) which also appeared in the perivascular spaces which normally contain developing granulocytic cells. Haemopoietic stem cells are almost entirely absent within these vessels. Those which were seen (fig. 8.4E) had a distorted appearance, suggesting cellular damage.

The severe cell depletion which occurred in the blood islands of these recipients may be compared with the much less severe changes which occurred in the yolk sac of embryos from control groups which were not inoculated with allogeneic blood early in embryonic life. Fig. 8.3B and fig. 8.4B are sections from the yolk sac of a 13 day old AA embryo which was inoculated at day 5 and day 6 with syngeneic adult blood before challenge at 10 days with adult allogeneic blood. The periarterial capillaries are packed with stem cells and their more differentiated derivatives. Many of these cells, however, have a ragged or crenulated appearance which suggests that some cell damage has occurred. Extreme cell depletion similar to that in figs. 8.3C, 8.4C, 8.3E and 8.4E was not observed. The cell damage in this case appears to be the result of the challenge at 10 days with adult allogeneic blood.

Blood islands in the yolk sac of 13 day old embryos inoculated at 10 days with adult allogeneic blood with no early treatment also show some cell damage (figs. 8.3D, 8.3F, 8.4D and 8.4F). The cells are not as tightly packed together as normally and severely depleted areas occur sporadically throughout the yolk sac fold. A single inoculation of adult allogeneic blood given at 10 days is

Fig. 8.3F Yolk sac from a 13 day old randomly bred chick embryo inoculated at 10 days with blood from an adult randomly bred donor. Some depletion of stem cells has occurred within the venous capillaries (v) surrounding the central artery (a). (e) marks the epithelium.

Azure II/methylene blue Magnification 90 x.

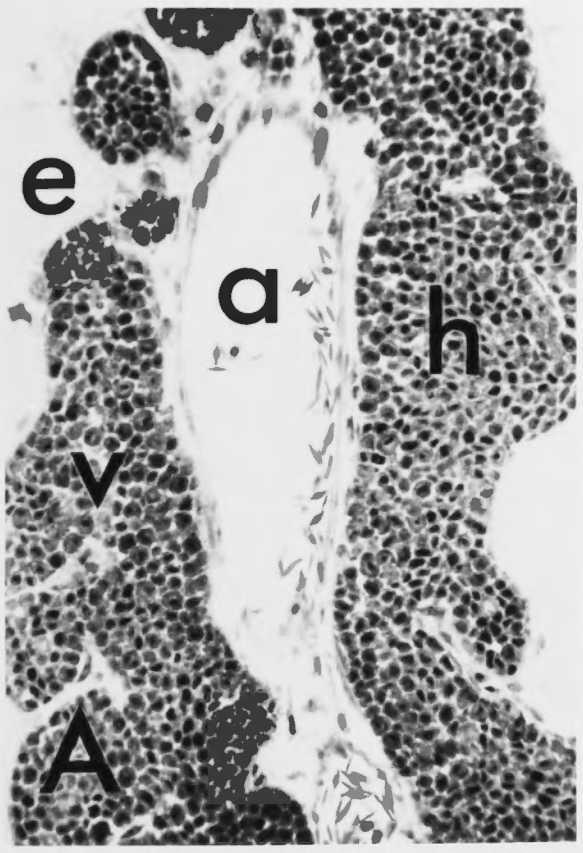
Fig. 8.3A Section through the yolk sac fold of a normal 13 day old randomly bred chick embryo. The periarterial venous capillaries (v) around the central artery (a) are filled with tightly packed haemopoietic stem cells and their more differentiated derivatives (h). (e) marks the yolk sac epithelium.
Azure II/methylene blue Magnification 90 x.

Fig. 8.3B Yolk sac fold from a 13 day old AA chick embryo inoculated at day 5 and day 6 with adult AA blood before challenge at day 10 with blood from a randomly bred donor. The periarterial venous capillaries (v) are filled with cells which have a ragged or crenulated outline suggesting that cell damage has occurred. (a) marks the central artery and (e) marks the yolk sac epithelium.
Azure II/methylene blue Magnification 90 x.

Fig. 8.3C Severe cell depletion within the blood islands of the yolk sac from a 13 day old randomly bred chick embryo inoculated at day 5, day 6 and day 10 with adult AA blood. The periarterial venous capillaries (v) contain only a few cells at the edges of the vessel. (e) marks the yolk sac epithelium.
Azure II/methylene blue Magnification 90 x.

Fig. 8.3D Yolk sac fold from a 13 day randomly bred chick embryo inoculated at day 10 with blood from an adult randomly bred donor. Developing cells within the venous capillary vessels (v) which surround the central artery (a) are less tightly packed than in normal embryos.
Azure II/methylene blue Magnification 90 x.

Fig. 8.3E Yolk sac from a 13 day old randomly bred chick embryo inoculated at day 5 and day 6 with adult AA blood and challenged at 10 days with blood from an adult randomly bred donor. The central artery (a) and the surrounding venous capillary plexus (v) are congested with mature red cells which are also seen in the perivascular space (r) between the yolk sac epithelium (e) and the venous capillaries. No stem cells can be identified.
Azure II/methylene blue Magnification 90 x.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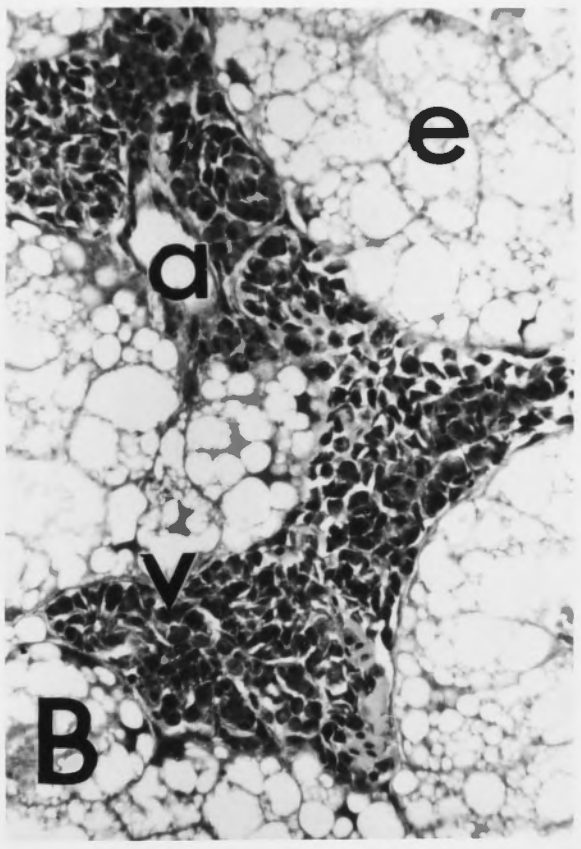
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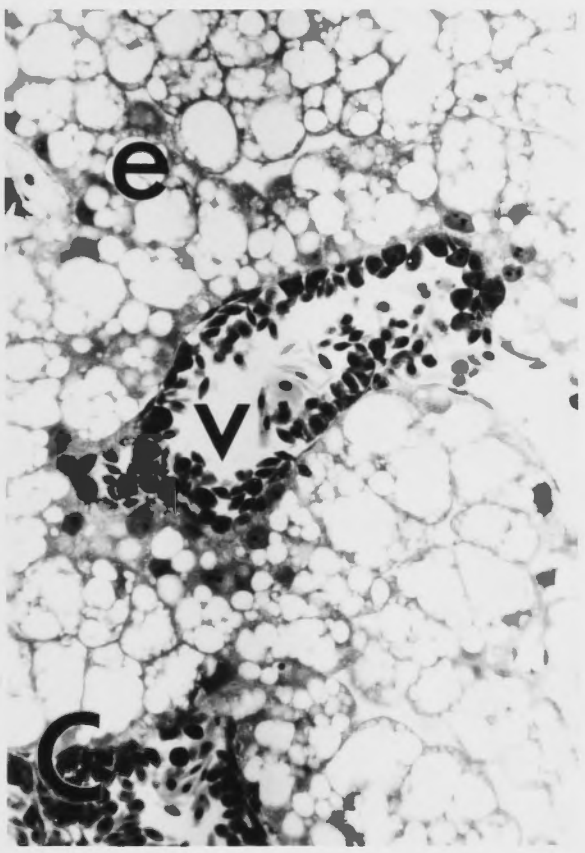
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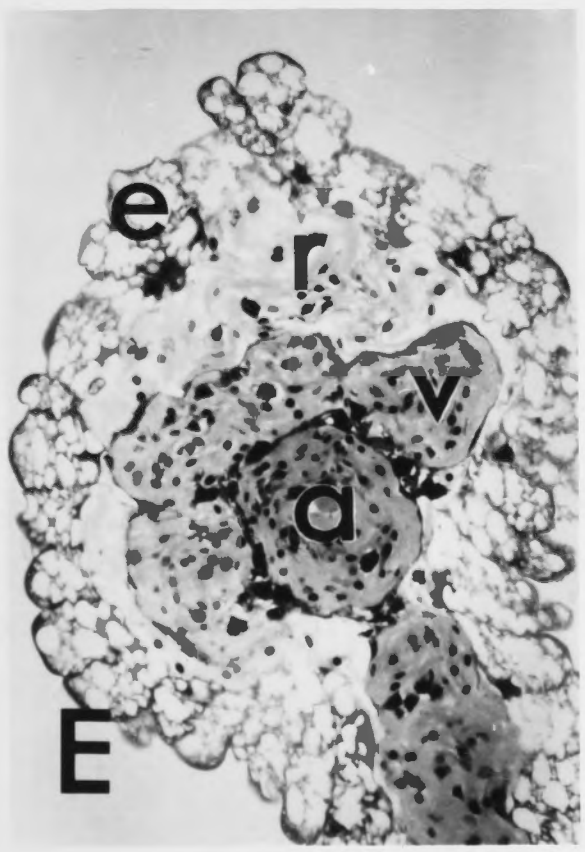
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Fig. 8.4F A periarterial venous capillary from the yolk sac of a 13 day old randomly bred chick embryo inoculated at 10 days with blood from an adult randomly bred donor. The vascular lumen (l) contains few haemopoietic stem cells.
Azure II/methylene blue Magnification 900 x.

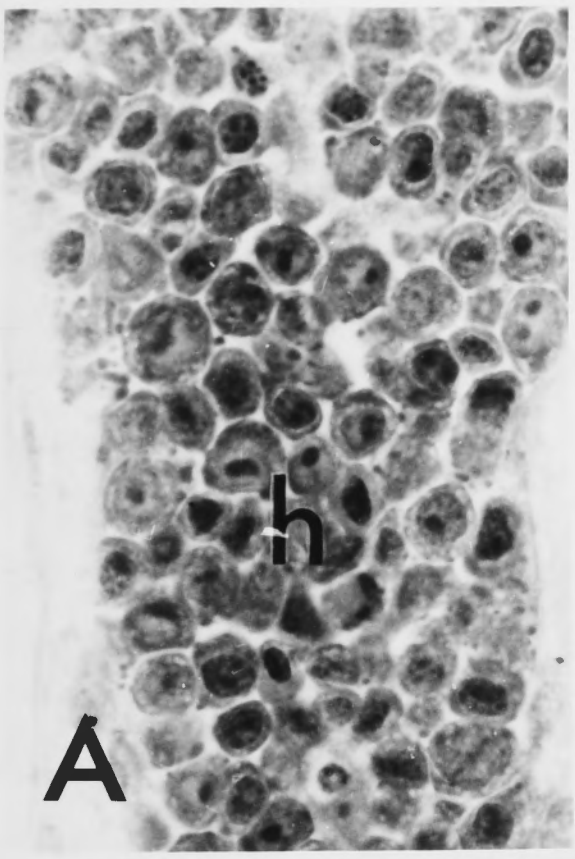
Fig. 8.4A Intense haemopoietic activity (h) within a periarterial venous capillary from a normal 13 day old randomly bred chick embryo.
Azure II/methylene blue Magnification 900 x.

Fig. 8.4B Periarterial venous capillary from a yolk sac fold of a 13 day old AA chick embryo inoculated at day 5 and day 6 with adult AA blood before challenge at day 10 with blood from an adult randomly bred donor. The vessel contains darkly staining cells (d) with a ragged outline which suggests that degenerative changes have occurred. (e) marks the epithelium.
Azure II/methylene blue Magnification 900 x.

Fig. 8.4C A periarterial venous capillary vessel from the yolk sac of a 13 day old randomly bred chick embryo inoculated at day 5, day 6 and day 10 with adult AA blood. The vascular lumen (l) contains few haemopoietic stem cells (h) or their early derivatives. (e) marks the epithelium.
Azure II/methylene blue Magnification 900 x.

Fig. 8.4D A periarterial venous capillary vessel from the yolk sac of a 13 day old randomly bred chick embryo inoculated at day 10 with blood from an adult randomly bred donor. The haemopoietic cells (h) within the vascular lumen are no longer packed together tightly as in a normal embryo of the same age (fig. 8.4A). A few mature red blood cells (r) are found within these vessels. (e) marks the epithelium.
Azure II/methylene blue Magnification 900 x.

Fig. 8.4E Darkly staining cells (d) with a crenulated appearance within a periarterial venous capillary from the yolk sac of a 15 day old randomly bred chick embryo inoculated at day 5 and day 6 with adult AA blood and challenged at day 10 with blood from an adult randomly bred donor. Mature red cells (r) are also found within the vascular lumen.
Azure II/methylene blue Magnification 900 x.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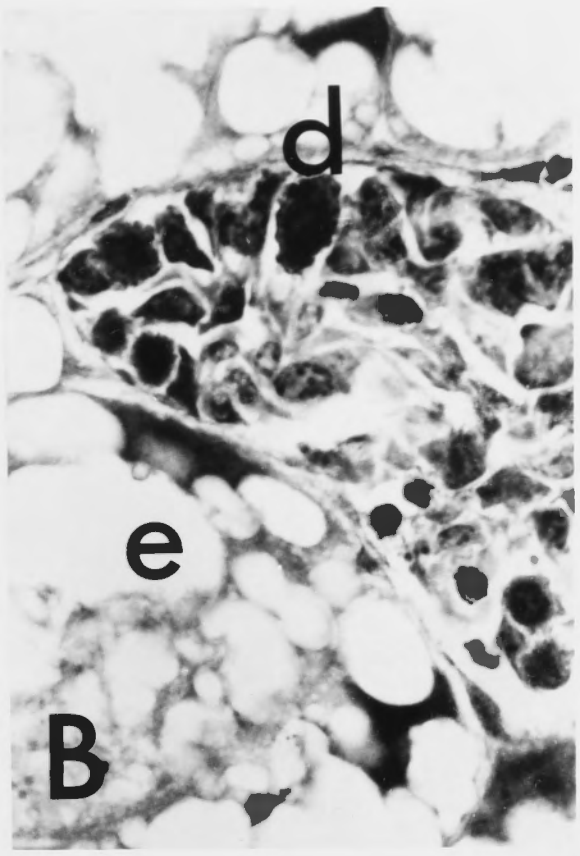
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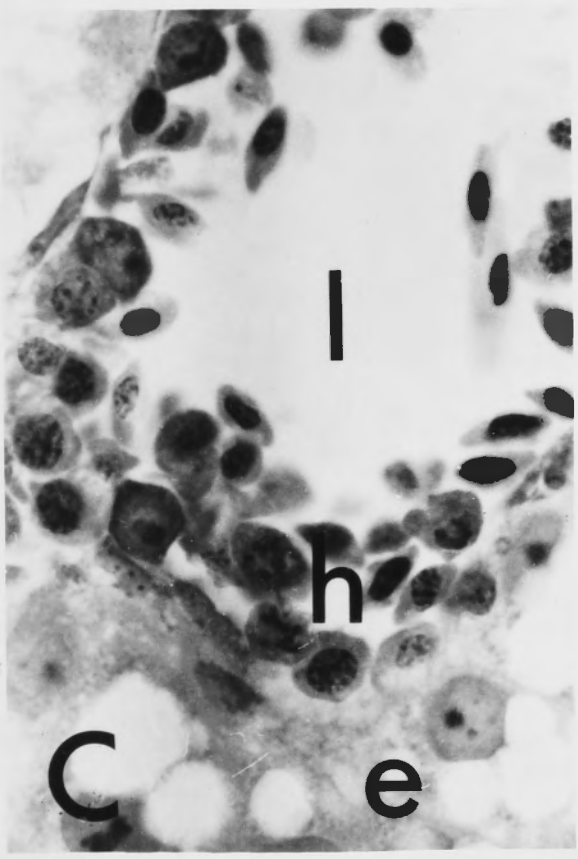
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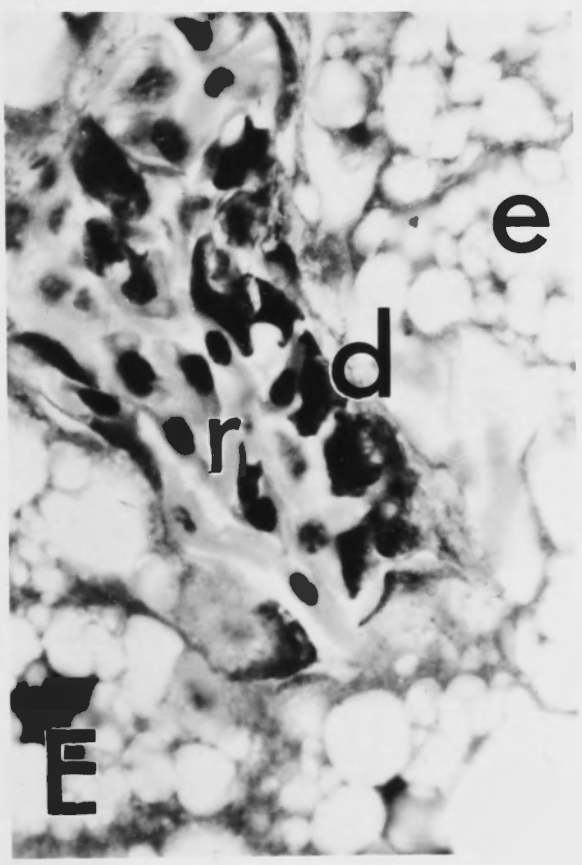
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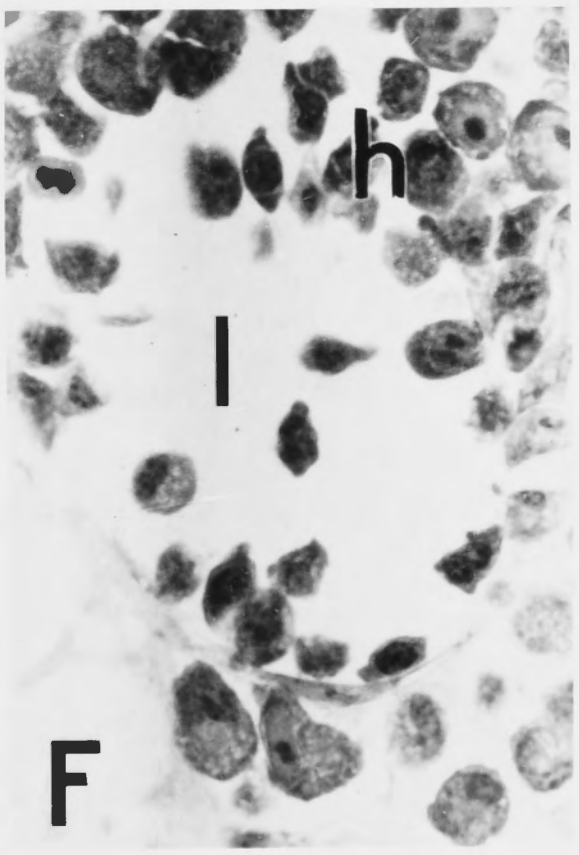
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thus sufficient to cause cell damage in the yolk sac, although cell depletion in the blood islands is not as severe as in younger embryos.

The inoculation of syngeneic adult blood onto the CAM of 10 day old AA embryos did not produce any depletion of haemopoietic stem cells in the blood islands and sections from the yolk sacs of these recipients at 13 days were essentially similar in appearance to those shown in figs. 8.3A and 8.4A.

The effect of the inoculation of adult allogeneic blood on the development of the yolk sac in these experiments is summarised in table 8.3. Early treatment with allogeneic adult blood, severely depleted the blood islands of the yolk sac, while inoculation of adult allogeneic blood at 10 days without early treatment caused a relatively small degree of cellular destruction.

Table 8.3

The effect of multiple inoculations of adult blood on haemopoietic activity in the blood islands of the yolk sac.

Embryo recipients	Early treatment at 5 and 6 days	Challenge at 10 days	Depression of haemopoiesis in the yolk sac
Randomly bred	Adult AA blood	Adult AA blood	severe
Randomly bred	——	Adult AA blood	slight
Randomly bred	Adult AA blood	Adult donor 1*	severe
AA	——	Adult donor 1*	slight
AA	Adult AA blood	Adult donor 2*	slight
		Adult donor 2*	slight
AA	——	Adult AA blood	none

*Blood from randomly bred donors which reacted against AA recipients.

Changes in the bursa after multiple inoculation of adult blood

The bursa of each embryo in these experiments was examined by light microscopy. Inoculation of adult allogeneic blood into the early embryo, either as an early treatment or as a single inoculum given at 10 days greatly decreased the number of haemopoietic stem cells within the bursal epithelium at 13 days.

As described in chapter 7, clusters of haemopoietic stem cells beneath the bursal epithelium are readily found from 12 to 14 days and many are seen infiltrating the bursal epithelium. This may be compared with the section shown in fig. 8.5A which is taken from the bursa of a 13 day old randomly bred embryo after inoculation with adult allogeneic blood at 5, 6 and 10 days. No haemopoietic stem cells can be seen, either beneath the bursal epithelium or insinuated between the epithelial cells. There is also no evidence of epithelial proliferation to form epithelial buds. This section is typical of the appearance of the bursa in all recipients which received adult allogeneic blood as an early treatment or as a challenge.

Changes in the thymus after multiple inoculation of adult blood

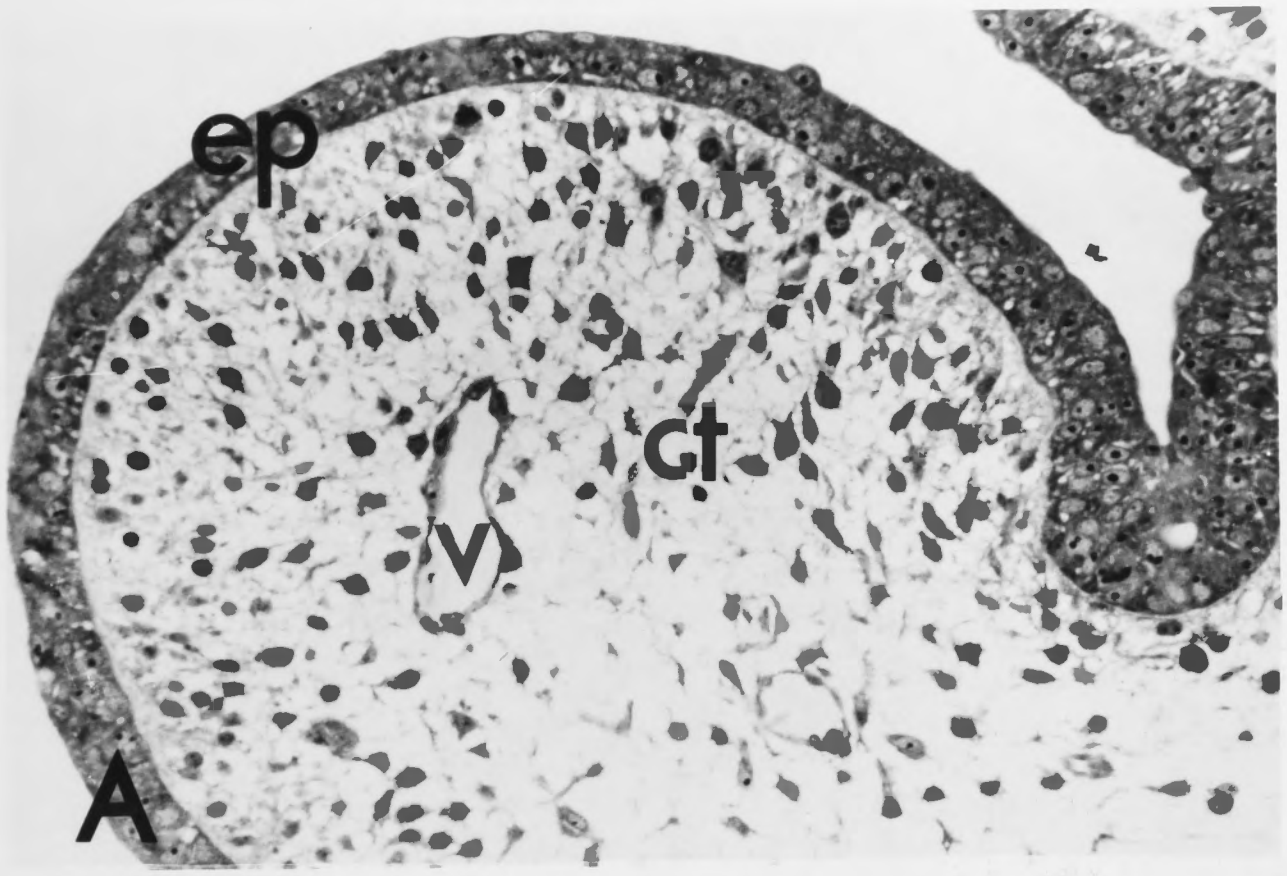
Early treatment with adult allogeneic blood was found to produce various degrees of aplasia in the thymus as shown in figs. 8.5B and 8.5C. These sections are taken from the thymus of a 13 day old randomly bred embryo, inoculated with adult allogeneic blood at 5, 6 and 10 days incubation. Although the thymic lobule is of normal size (fig. 8.5B) lymphopoiesis is greatly reduced. In comparison with the appearance of a normal thymus at 12 or 14 days incubation (chapter 7) only a few cells with the heavily chromatic nucleus characteristic of developing thymocytes (Venzke, 1952), are scattered throughout the tissue. Large, pale-staining cells, with the appearance of thymic epithelial cells (Venzke, 1952) are abnormally evident instead.

This depression in the number of developing thymocytes was only characteristic of experimental groups which had been given early treatment with adult allogeneic blood.

Fig. 8.5A Transverse section through the bursa of a 13 day old randomly bred chick embryo inoculated at day 5 and day 6 with adult AA blood before challenge at 10 days with blood from an adult randomly bred donor. No haemopoietic stem cells could be found within the bursa, either infiltrating the bursal epithelium (ep) or in the underlying connective tissue layer (ct) which contains small blood vessels (v).
Azure II/methylene blue Magnification 325 x.

Fig. 8.5B Transverse section through the thymus of a 13 day old randomly bred chick embryo inoculated at day 5 and day 6 with adult AA blood before challenge at 10 days with blood from an adult randomly bred donor. There is little lymphopoiesis within the thymic lobe (t). (ct) marks the surrounding connective tissue.
Azure II/methylene blue Magnification 335 x.

Fig. 8.5C Detail from the section shown in fig. 8.5B to show the poor lymphoid development in the thymus of this recipient. Although a few developing lymphoid cells may be identified (l), large pale cells of apparent epithelial origin (e) predominate.
Azure II/methylene blue Magnification 1,300 x.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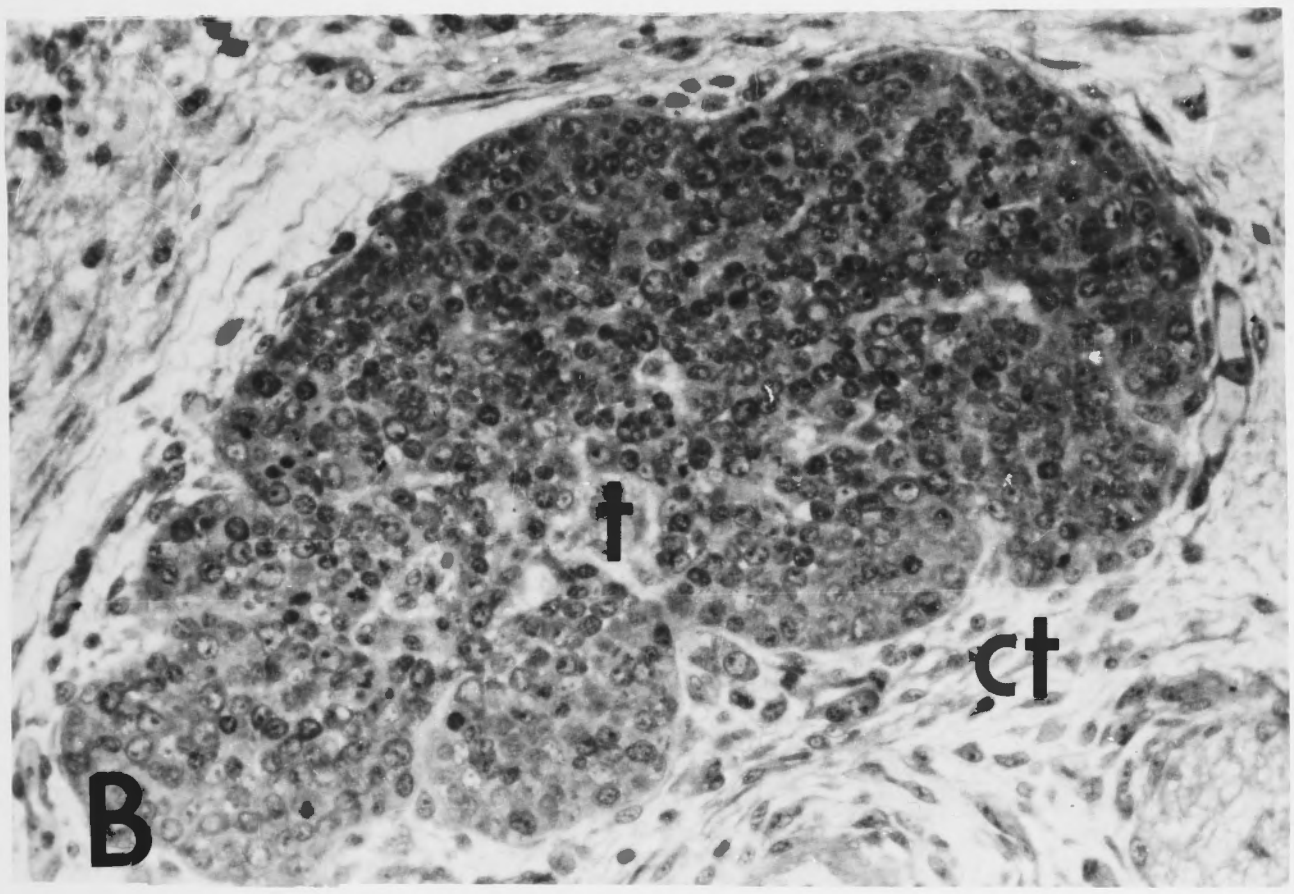
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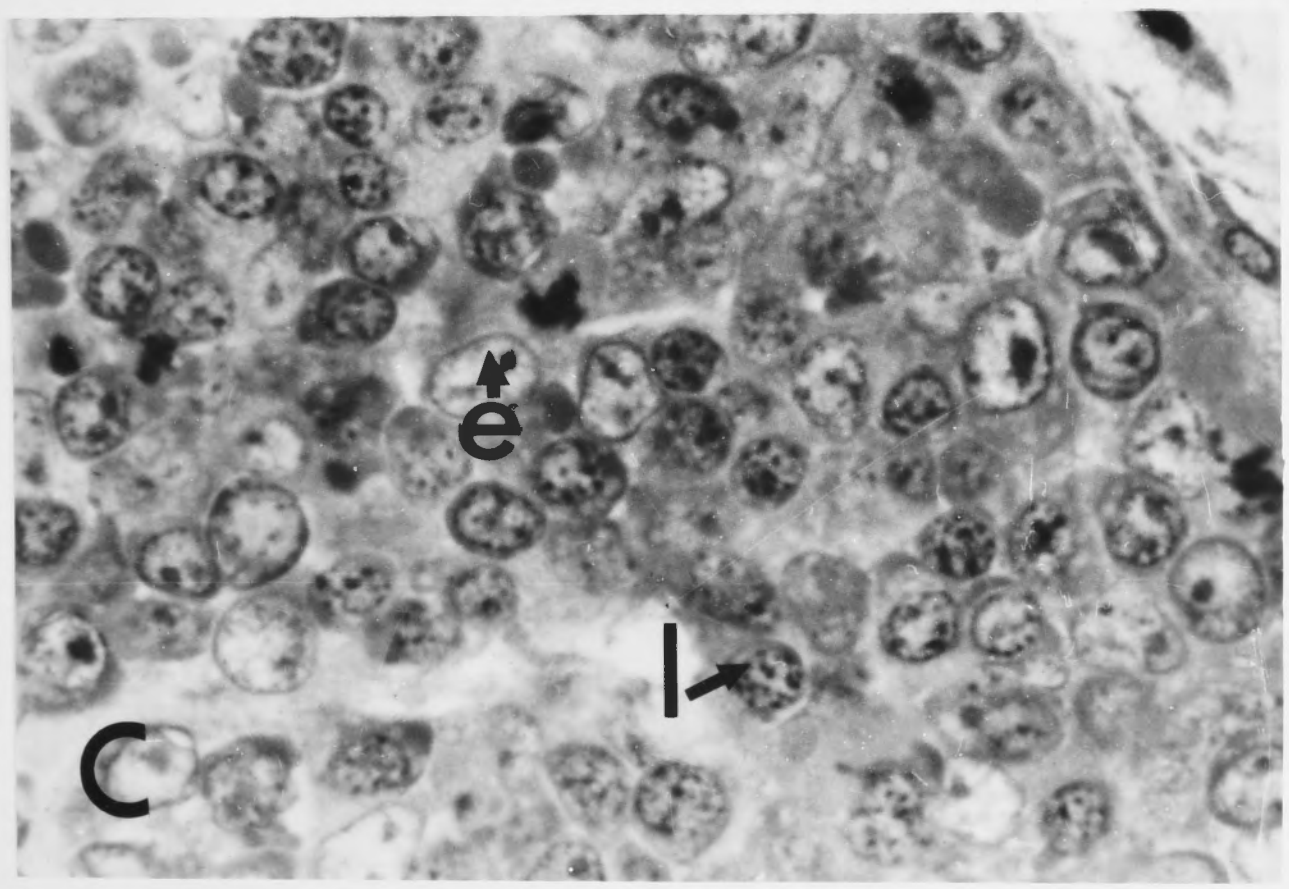
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Table 8.4

The effect of multiple inoculations of adult blood on the development of the thymus and bursa.

Embryo recipients	Early treatment at 5 and 6 days	Challenge at 10 days	Effect on the number of haemopoietic stem cells within the bursal epithelium at 13 days	Effect on the thymus at 13 days
Randomly bred	Adult AA blood	Adult AA blood	Very few	Thymic aplasia
Randomly bred	——	Adult AA blood	Very few	Normal/Proliferative lesions
Randomly bred	Adult AA blood	Adult donor 1*	Very few	Thymic aplasia
AA	——	Adult donor 1*	Very few	Normal/Proliferative lesions
AA	Adult AA blood	Adult donor 2*	Very few	Normal/Proliferative lesions
AA	——	Adult donor 2*	Very few	Normal/Proliferative lesions
AA	——	Adult AA blood	Normal	Normal

*Blood from randomly bred donors which react against AA recipients.

Embryos which were given allogeneic blood at 10 days without early treatment or after early treatment with syngeneic blood showed normal thymic development or small proliferative lesions similar to those described in chapter 7.

The effect of the multiple inoculation of adult blood on the thymus and bursa of recipient chick embryos is summarised in table 8.4.

Changes in haemostasis during a GVHR

The preceding experiments have shown that haemorrhagic lesions which have been described in chapter 4 can also be induced in randomly bred embryos by multiple inoculations of adult allogeneic blood. Further tests were made to determine the effect of this condition on haemostasis.

Randomly bred embryos were inoculated under the shell membrane lining the air space at 5, 6 and 10 days with 0.1 ml inoculations of a 1:1 dilution of adult AA blood in Alsever's solution. Control embryos were inoculated in a similar manner with 0.1 ml of Alsever's solution. At 13 days bleeding time and clotting time of blood removed from an allantoic vein (chapter 2) were measured for at least 6 embryos from both the experimental and the control groups. The spleens of these embryos were weighed to determine the degree of splenomegaly induced by the GVHR. The results of these experiments are shown in table 8.5.

Table 8.5

Bleeding time, clotting time and spleen weights of 13 day randomly bred embryos after inoculation at 5, 6 and 10 days with diluted AA adult blood. Mean values with the standard error are given.

	Experimental group	Control group
Bleeding time (seconds)	200 ± 30	95 ± 6
Clotting time (minutes)	41 ± 7	12 ± 2
Wet weight of the spleen (mg)	9.8 ± 0.8	9.9 ± 0.6

As can be seen, bleeding time as well as clotting time was greatly increased in the experimental animals and the spleen was also not enlarged.

Discussion

As previously described, a GVHR in very young chick embryos is characterised by cell destruction in the blood islands of the yolk sac, aplasia of intra-embryonic haemopoietic tissues and superficial haemorrhages. Similar changes were observed in randomly bred embryos inoculated at day 5 and again at day 6 with adult allogeneic blood and then challenged at day 10 with another inoculum of adult allogeneic blood. At 13 days, surviving embryos had haemorrhages, few haemopoietic stem cells remained in the blood islands, lymphopoiesis in the thymus was depressed and the infiltration of stem cells into the bursal epithelium appeared to have been prevented.

Embryos which were not inoculated with adult allogeneic blood until day 10 still showed degenerative changes in the yolk sac which suggests that the destruction of primitive haemopoietic stem cells during a GVHR is not confined to early embryonic life. In 10 day recipients, proliferative lesions developed in the thymus, but did not occur in the bursa, a result which may be related to the time at which stem cells migrate into these tissues, as discussed in chapter 7.

The development of haemorrhagic lesions during an early GVHR was found to correlate with changes in the haemostatic properties of the blood, both the bleeding time and the clotting time were unusually long. The most likely explanation for these changes is thrombocytopenia following precursor cell destruction in the yolk sac, complicated by a slow development of the blood coagulation system, which even in normal embryos does not develop until 12 days (Pickering and Gladstone, 1925; Kane and Sizer, 1953).

The development of thrombocytopenia during a GVHR has been reported by Oliner and co-workers (1961) in F_1 hybrid mice inoculated with parental spleen cells and by Miller (1967) in a child with thymic dysplasia who developed GVHR after a bone marrow transfusion. Stasny and co-workers (1963) also attributed the haemorrhagic lesions which devel-

oped in the skin of adult rats during a GVHR to thrombocytopenia. In comparison, the increase in blood clotting time during a GVHR, appears to be confined to very young chick embryos. Experiments in older chick embryos indicate that the development of a GVHR later in embryonic life, may decrease the clotting time by causing the thromboplastin content of tissues such as the spleen, to increase (Zaleski, 1968; Mann et al., 1969).

The experiments described in this chapter have demonstrated that the development of an early GVHR specifically prevents the subsequent development of proliferative lesions such as splenomegaly or pock formation. This effect could not be produced by the early inoculation of adult syngeneic blood and could not be attributed to an early colonization of the host by donor cells. The results support the hypothesis that the host component involved in the development of proliferative lesions is either the yolk sac derived stem cell or one of its early more differentiated derivatives. The early GVHR appears to prevent the migration of stem cells from the yolk sac into the developing embryo and therefore also prevents any subsequent development of proliferative lesions.

CHAPTER 9

DISCUSSION

CHAPTER 9. DISCUSSION

The graft-versus-host reaction was initially conceptualised as a response of donor cells against foreign transplantation antigens of the host (Simonsen, 1957, 1962; Billingham and Brent, 1957). However, as discussed in chapter 1, further studies have indicated that cells of the host are also involved. The GVHR can now be considered as a reaction in which allogeneic T-lymphocytes (Cooper et al., 1966) interact with host cells of haematogenous origin (Ramseier and Billingham, 1966; Elkins and Guttman, 1968; Lafferty and Jones, 1969; Streilein and Billingham, 1970a, b; Elkins, 1971). The chick embryo provides a convenient system in which to study this interaction with the host components, since the effect of donor cells on the different elements of the host haemopoietic tissue at various stages of embryonic differentiation can be examined with ease.

The primary effect of the GVHR in very young chick embryos is the destruction of haemopoietic stem cells in the blood islands of the yolk sac, which during early embryonic life constitute the sole haemopoietic tissue (Lemež, 1964). Destruction of these cells appears to prevent the migration of stem cells from the yolk sac into the primordia of intra-embryonic haemopoietic tissues which therefore remain aplastic. Other lesions observed during GVHRs in these very young embryos are widespread superficial haemorrhages. Although the cause of this vascular damage has not been clearly established it may be related to the release of various cell breakdown products from the yolk sac into the circulating blood, as reflected by a rise of lysosomal enzyme activity in the plasma. Blood clotting mechanisms appear to be insufficiently developed to stop bleeding and cell destruction in the yolk sac presumably produces thrombocytopenia and thus prevents thrombus formation.

The pathogenesis of a GVHR initiated later in embryonic life can be related to the migration of yolk sac derived stem cells into the developing lymphoreticular organs. Typical proliferative lesions develop in those tissues which contain stem cells when the donor inoculum is introduced, while aplasia was seen in other lymphoreticular organs which had not yet been colonised by stem cells.

The results indicate that the pathogenesis of a GVHR in the chick embryo depends on the reaction of two types of host cell, primitive haemopoietic stem cells in the yolk sac which are destroyed or inactivated and yolk sac derived stem cells within the embryonic lymphoreticular tissue which are stimulated to proliferate. Since only primitive stem cells in the yolk sac are affected during the GVHR in very young embryos, but both types of cell are affected in older embryos, the pathological changes which characterise a GVHR closely reflect the development of the host's haemopoietic tissue.

The basis for the dual effect of donor cells on the host haemopoietic tissue has not been established. It seems likely that a functional difference between primitive haemopoietic stem cells and yolk sac derived stem cells is involved rather than an environmental difference between the yolk sac and other haemopoietic tissues. Functional differences between various classes of stem cell have been demonstrated experimentally in the mouse (McCulloch, 1970; McCulloch and Till, 1970) and may occur in the chick embryo.

In the mouse, the haemopoietic system contains a class of pluripotential stem cells (McCulloch and Till, 1970) which are identified by their ability to form spleen colonies in heavily irradiated recipients (Till and McCulloch, 1961). They can give rise to granulocytes, erythroblasts, megakaryocytes, thymocytes and lymph node cells (Fowler et al., 1967; Curry et al., 1967; Wu et al., 1968b) and are also capable of self renewal (Siminovitch et al., 1963). Progeny of these pluripotential stem cells are early committed stem cells (McCulloch and Till, 1970) which give rise to cells of only one developmental pathway.

Granulocytic cells develop from stem cells which are closely related to the pluripotential stem cell (Wu et al.,

1968a) and which give rise to colonies in culture (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966). Erythrocytic cells derive from another early committed stem cell which is sensitive to erythropoitin (Bruce and McCulloch, 1964; McCulloch and Till, 1970). Both types of stem cell can be separated from pluripotential stem cells by physical means (Worton et al., 1969; Moore et al., 1970; Haskill et al., 1970; Stephenson and Axelrad, 1971).

Analogous cell types may exist in the haemopoietic system of the chick embryo. Primitive stem cells in the chick embryo yolk sac can give rise to erythrocytes, thrombocytes and granulocytes (Danchakoff, 1908, 1916c; Sugiyama, 1926; Edmonds, 1966) and are capable of repopulating both lymphoid and myeloid tissues of the irradiated chick embryo (Moore and Owen, 1967c). They may be analogous to pluripotential stem cells in the mouse which are also destroyed during a GVHR (Davis and Cole, 1967; Davis et al., 1970). Similarly, yolk sac derived stem cells which are stimulated to proliferate during a GVHR may correspond to early committed stem cells. The analogy is strengthened by the fact that proliferation of in vitro colony forming cells is increased in neonatal F_1 mice with GVH disease induced by the injection of parental spleen cells (Metcalf and Moore, 1971).

The manner in which primitive haemopoietic stem cells are destroyed or inactivated during a GVHR has not yet been established. The development of proliferative lesions, however, appears to involve some form of direct stimulation between host and donor cells. Experiments on pock formation in the chick embryo (Killby, Lafferty and Ryan, in press) have shown that division of embryonic cells is required to initiate pock formation. Donor cells, however, do not need to divide, although they must be viable. These results suggest that in a GVHR donor cells directly stimulate embryonic cells to divide.

The pathological changes which develop in the chick embryo during a GVHR may be compared to those which have been described in other animals. Since very early embryos were not generally examined, most descriptions of GVHRs refer to proliferative lesions in the lymphoid tissue rather than

aplasia (Simonsen, 1962). However, a few cases are recorded in which a GVHR apparently developed during embryonic life. A wasting syndrome, resembling a mild GVHR, developed in rats from a backcross mating which were compatible with the mother at the Ag-B locus (Palm, 1970). Since the thymus of these animals showed marked acellularity and other lymphoid tissues were abnormally developed, the condition may be similar to GVHRs in very young chick embryos. Another instance in which maternal cells appear to have crossed the placenta during embryonic life has been described by Kadowaki and coworkers (1965) in a male child who had XX/XY chimaerism in the blood. The boy, who died at 16 months, had a poorly developed thymus and almost no lymphoid tissue associated with the gut. The spleen and lymph nodes, however, had become enlarged.

A GVHR in very young chick embryos, in which the development of lymphoreticular tissues is prevented, is clinically similar to reticular dysgenesia, a condition which occurs in humans when primitive haemopoietic stem cells fail to give rise to more differentiated derivatives (Hoyer et al., 1968). The lymphoreticular tissue of these children also remains aplastic (de Vaal and Seynhaeve, 1959; Gitlin et al., 1964).

In many graft-versus-host reactions described in adult or neonatal animals, donor cells appear to have the same dual effect on the host's haemopoietic tissue as they do in the chick embryo. The development of proliferative lesions is frequently accompanied by atrophic changes which may be due to the destruction of primitive stem cells. As mentioned previously, Davis and coworkers (1967, 1970) have shown that colony-forming units in the spleen and bone marrow of F_1 hybrid mice are destroyed by the injection of parental lymphoid cells.

In several species, myeloid hypoplasia in the bone marrow has been described during a GVHR, with the disappearance of immature granulocytes and erythrocytes (Nisbet et al., 1960; Nisbet and Heslop, 1962a, b; Billingham et al., 1962; Hathaway et al., 1966; Githens et al., 1968). This may be comparable to the cell depletion which occurs in the blood islands of the chick embryo yolk sac during a

GVHR. The destruction of primitive stem cells during a GVHR in adult or neonatal animals may account for the biphasic nature of lymphoid tissue lesions (McBride, 1966). These are generally characterised by a proliferative phase (Simonsen, 1962; Billingham et al., 1962; Arakawa et al., 1966; Hathaway et al., 1967) followed by varying degrees of atrophy and fibrosis (Howard, 1961).

Since the development of the haemopoietic tissue has a major effect on the pathogenesis of the GVHR in the chick embryo, it might be expected that the GVHR in neonatal or adult animals might be even more complex, due to the presence of haematogenous elements absent in the embryo. Descriptions of GVHRs in various neonatal or adult animals suggest that this is the case. Changes have been reported which do not occur in young embryos. For example, few proliferating donor cells can be detected in the spleen of very young chick embryos during a GVHR (Nisbet and Simonsen, 1967; Weber, 1970), however, spleen enlargement in chick embryos examined shortly before hatching involves a considerable donor component (Owen et al., 1965; Nisbet and Simonsen, 1967). The difference in donor cell activity during spleen enlargement in young and in older chick embryos is reflected in the different pathological changes which occur in the spleen at each stage (Isacson, 1959). Donor cell proliferation is apparently also a general feature of GVHRs in neonatal mice (Zeiss and Fox, 1963; Nakić et al., 1967), F_1 hybrid mice injected with parental cells (Howard et al., 1961; Fox, 1962), neonatal rats (Nowell and Defendi, 1964) and of local GVHRs in the kidney of adult rats (Elkins, 1970).

Since the graft-versus-host reaction and the mixed lymphocyte reaction have similar genetic and other prerequisites, Wilson and Elkins have suggested (as reported by Billingham, 1971) that an in vivo mixed lymphocyte reaction may play an important role in the development of a GVHR. The ability of cells to stimulate in an in vitro mixed lymphocyte reaction is a T-lymphocyte function (Maclaurin, in press). If a similar type of stimulation can occur in vivo, donor cell proliferation during a GVHR may depend on the presence of mature thymus-derived lympho-

cytes in the host. The absence of T-lymphocytes in early embryonic life may thus explain the absence of donor cell proliferation during a GVHR in young chick embryos. This is supported by the finding that donor cell proliferation in the chick embryo spleen during a GVHR first increases at 17 days (Nisbet and Simonsen, 1967), the time when immunocompetence can also first be detected (Seto, 1971).

Another feature of the GVHR in adult or neonatal recipients which may be less prominent in embryonic animals is the cytolytic activity of donor cells against the host as described by Singh and coworkers (1971), Gengozian and Hübner (1971) and by Cerottini and coworkers (1971). Experiments in adult irradiated mice injected with allogeneic spleen cells have shown that two types of donor cells can express cytolytic activity against the host (Cerottini et al., 1971). These are plaque-forming cells and cytotoxic lymphocytes. Plaque-forming cells lyse target tissue by the release of humoral type antibody while cytotoxic lymphocytes express a form of cell mediated immunity which can not be affected by antisera against immunoglobulin (Chapuis and Brunner, 1971).

Although the cytolytic effects of donor cells in embryonic animals have not yet been studied in detail, antibody formation by donor cells has not been detected during GVHRs in chick embryos (Macpherson and Deamer, 1965; Lafferty and Jones, 1969). In comparison, a Coombs positive haemolytic anaemia develops in newly hatched chicks inoculated 3 or 4 days before hatching with adult spleen cells or blood (Simonsen, 1957; Macpherson and Deamer, 1965). A similar condition has also been described in neonatal rabbits undergoing a GVHR (Porter, 1960c).

It has been suggested that the absence of antibody formation by donor cells in embryonic recipients may be due to the immaturity of the macrophage system (Lafferty and Jones, 1969). It may also reflect the absence of lymphocytes in the embryonic host, since it appears from studies in a variety of experimental systems, that the stimulation of the immune system with allogeneic lymphocytes can have an adjuvant effect on the subsequent response to unrelated antigens. For example, the work of Katz and coworkers (1971a, b) has shown that the introduction of allogeneic

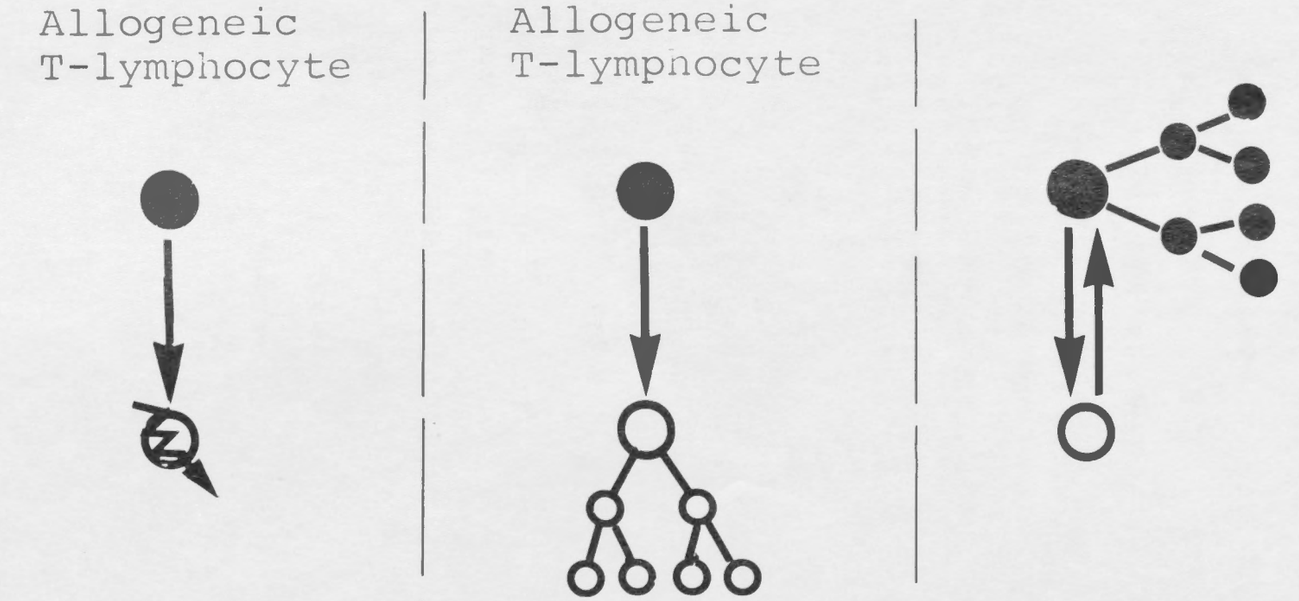
Allogeneic T-lymphocyte
Allogeneic T-lymphocyte
Primitive haemopoietic stem cell
DESTROYED
More specialised stem cell
STIMULATED TO PROLIFERATE
Mature T-lymphocyte
STIMULATES
GVHR in early embryos
GVHR in later embryos
GVHR in an immunocompetent animal

Diagram 9.1

lymphocytes into guinea pigs primed with a hapten-carrier complex, prepares them for a striking secondary response to the hapten when it is later presented on another carrier. McCullagh (1970) has shown that tolerance to sheep red blood cells in rats is abrogated by the inoculation of allogeneic lymphocytes and Ekpaha-Mensa and Kennedy (1971) found that the presence of irradiated lymphoid cells in the culture medium dramatically increased the plaque-forming cell response to the same antigen.

If a similar adjuvant effect operates in a graft-versus-host situation, the production of antibody against the host by the donor cells may be stimulated by the presence of allogeneic lymphocytes of the host. This effect may therefore account, in part, for the production of antibody during a GVHR in adult or neonatal recipients (Simonsen, 1957; Feldmann and Yaffe, 1958; Porter, 1960c; Gengozian and Hübner, 1971) while this does not occur in embryonic recipients in which lymphocytes have not differentiated (Macpherson and Deamer, 1965; Lafferty and Jones, 1969).

The pathogenesis of a GVHR in neonatal or adult animals thus appears to differ in several respects from the pathogenesis of a GVHR in embryonic recipients. These differences may be related to the presence of mature lymphocytes in neonatal and adult recipients. The GVHR may therefore be considered not as a single disease, but as a series of related pathological conditions, each based on an interaction between donor cells and different haematogenous elements of the host. The GVHR thus becomes more complex as the haemopoietic system of the host matures.

The increase in complexity of the graft-versus-host reaction in the developing embryo is illustrated in diagram 9.1. The reaction can be divided into three components :

(1) The destruction or inactivation of primitive haemopoietic stem cells by allogeneic T-lymphocytes. This is the only reaction to occur in very young embryos in which more specialised stem cells have not yet differentiated.

(2) The stimulation of a more specialised type of stem cell to proliferate within the lymphoreticular tissues of the host. This reaction occurs in older

embryos in which there is also a concomitant destruction of primitive stem cells.

(3) The development of an *in vivo* mixed lymphocyte reaction within an immunocompetent host.

Experiments on the MLR *in vitro* indicate that a reaction of this kind *in vivo* would involve a one way stimulation of donor cells when parental tissue is inoculated into an F_1 recipient (Wilson *et al.*, 1967), otherwise host lymphocytes, presumably, may also proliferate. Destruction of primitive stem cells and proliferation of more specialised stem cells appears to occur during the GVHR in immunocompetent animals. However, an additional spectrum of pathological changes may also occur, involving donor cell cytotoxicity and the production of antibody, changes which appear to be less important in the pathogenesis of the GVHR in embryonic animals.

Diagram 9.1 thus summarises the manner in which the development of the host haemopoietic system may govern the pathogenesis of a graft-versus-host reaction.

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